Asian Biomedicine Vol. 7 No. 1 February 2013; 39-49 DOI:10.5372/1905-7415.0701.149

Original article

Enhanced antitumor effect of axitinib synergistic interaction with AG490 via VEGFR2/JAK2/STAT3 signaling mediated epithelial-mesenchymal transition in cervical cancer *in vitro*

Rong-Rong Zhang[a,b], Hong Wang[a], Ning Hui[b], Ping Zhang[a]
[a]Department of Gynecology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, [b]Department of Gynecology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Background: Epithelial to mesenchymal transition (EMT) not only confers tumor cells with a distinct advantage for metastatic dissemination, but also it provides tumor cells for proliferation and chemotherapy resistance. Thus, inhibiting this process using single or multiple agents, remains a field of intensive research. The development of EMT has been implicated in not only conferring cancer cells distant metastasis, but also providing tumor cells advantages for proliferation and chemotherapy resistance.
***Objective*:** We investigated whether axitinib synergistic interaction with AG490 could effectively block the growth and EMT-mediated tumor metastasis in cervical cancer *in vitro*.
***Methods*:** We addressed synergism effects on tumor growth, axitinib, AG490 alone and in any possible combinations on cell viability, apoptosis and cell cycle distribution were evaluated by XTT assay, Annexin V/propidium iodide staining and flow cytometric analysis. To assess how combination therapy affected chemotactic motility in tumor cells, wound-healing migration and the Boyden chamber invasion assays were applied *in vitro*. Further, using western blots, the key signaling molecules and pathways in response to axitinib and AG490 combination treatment in anti tumor growth and anti EMT-mediated tumor metastasis were analyzed in Hela cells.
***Results*:** Therapy with axitinib and AG490 resulted in strong synergistic inhibition of proliferation, migration and cell cycle arrest without potently effect on apoptosis induction relative to AG490 monotherapy and control in *vitro*. Furthermore, phosphorylation of VEGFR2 (Tyr^{1175}), JAK2 ($Tyr^{1007/1008}$) and STAT3 (Tyr^{705}) was completely blocked in parallel with significant reduction expressions of N-cadherin and obvious enhancement levels of E-cadherin through down-regulating Snail responding to combination treatment in cervical cancer cells.
Conclusion: We first provided evidences that combination therapy of axitinib and AG490 significantly enhances anti-tumor effect via VEGFR2/JAK2/STAT3 signaling mediated growth arrest and EMT-mediated tumor metastasis inhibition, holds promise for an efficacious treatment of these advanced patients.

Keywords: AG490, axitinib, EMT, Hela cells, JAK2, STAT3, VEGFR2

Cervical cancer is the third most common malignancy in females, accounting for approximately 529,800 total new cases in 2008 worldwide [1]. Despite considerable advances achieved through systemic therapy, the long-term survival of epithelial cervical cancer still shows unfavorable. Recurrence and metastases remain a formidable obstacle for cervical cancer therapy and are a main cause of cancer mortality [2]. The majority of advanced cervix carcinomas are resistant to anticancer drugs and show unsatisfactory responsiveness to conventional chemotherapy drugs including cisplatin, 5-fluorouracil and gefitinib on patient survival rate [2]. Therefore, the control of such chemoresistance underscores the need for new strategies to develop effective treatments for cervical cancer.

Understanding the biology of local or regional metastasis present in a significant portion of patients

Correspondence to: Ping Zhang, MD, Department of Gynecology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China. E-mail: pzhang 2011@126.com and Ning Hui, MD, Department of Gynecology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China. E-mail: nhui2000@126.com

at the time of diagnosis is, therefore, important in identifying how cervical cancer cells acquire the ability to invade surrounding tissue. Recapitulation of the developmental process of epithelial to mesenchymal transition (EMT) has been proposed as a crucial mechanism for enabling cancer cell invasion and dissemination [3, 4]. Metastatic progression of cancer-associated EMT is characterized by morphologic changes from polarized epithelial tumor cells converted into motile fibroblast-like cells, which enable them to lose intercellular adhesion, invade the basement membrane beneath, enter blood vessels, and disseminate into secondary organ [5, 6]. More recently, EMT in cancer progression has been widely studied *in vitro and in vivo*. Numerous reports showed that inhibition of EMT progression contributed to tumor growth arrest and metastasis suppression as well as the improved cancer therapeutic effect.

Janus kinase (JAKs) is a non-receptor tyrosine kinase that plays an important role in cellular signaling process of tumorigenesis [7]. Among different JAKs, the JAK2 is of particularly interest. Engagement of cell surface cytokine receptors activates JAK2, which subsequently phosphorylates and activates latent cytoplasmic STAT3 protein to be active in dimerization, translocation to nucleus, regulation transcription of various target genes [8, 9]. Several kinases have been implicated in the phosphorylation of JAK2, especially for the predominant vascular endothelial growth factor receptor 2 (VEGFR2) system. Upon activation of VEGFR2/JAK2/STAT3 was responsible for cell function mainly through modulation cell survival, proliferation, metastasis, angiogenesis, inflammation and immune evasion in tumor microenvironment [10-12]. Recent published studies have documented an association between STAT3 and EMT [13, 14]. Yadav A et al reported that interleukin-6 promotes head and neck tumor metastasis by inducing epithelial-mesenchymal transition via activation JAK/STAT3 signaling, indicating that this pathway may be critical for cytokine and growth factor-mediated responses regulating EMT biology in fibrogenesis and cancer [13]. Thus, agents that disrupt this pathway would be good candidates for cancer therapy.

The tyrphostin AG490 was the first identified as one of the most promising classes of small molecule JAK2 inhibitors. It exhibits anti-tumor effects through the induction of apoptosis in multiple human tumor cell lines *in vitro* and against tumor cell invasion in some pre-clinical cancer models. However, the results of JAK2 inhibition-based strategies for treating human cancer alone have not met expectations observed in patients. This limited success is largely attributed to requirement of high concentrations for effective JAK2 inhibition and chemotherapy tolerance [15].

Axitinib (AG-013736) is a novel, selective, small-molecule inhibitor, which targets the vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3. Given the strong preclinical evidence for anti-angiogenic, anti-tumor, and anti-metastatic activity [16, 17], it is currently in phase I to III evaluation in a range of solid tumors including advanced renal cell carcinoma [18], thyroid cancer [19], non-small cell lung cancer [20], colorectal cancer [21] and melanoma [22]. Results from phase II studies show that axitinib is active in cytokine-refractory metastatic renal-cell cancer as well as improves progression-free survival and overall survival in advanced and metastatic pancreatic cancer when combined with gemcitabine [23]. In the light of the important roles of STAT3 signaling in tumor growth and metastatic progression of cancer-associated EMT, we hypothesized that the enhanced antitumor effects on human cervical cancer, as dual inhibition of VEGFR2 and JAK2 by combining therapy of axitinib and AG490 could be synergistic. Overall, our study may for the first time provide guidance in designing rational combination therapeutic strategies for patients with advanced cancers.

Materials and methods

Reagents and antibodies

A 10 mmol/L stock solution of AG490 and axitinib (Sigma, USA) was dissolved in DMSO and then stored at -20°C as small aliquots until needed. The antibodies anti-VEGFR2, anti-JAK2, anti-STAT3, phospho-specific anti-VEGFR2 (Tyr^{1175}), anti-JAK2 ($Tyr^{1007/1008}$) and anti-STAT3 (Tyr^{705}) were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies against E-cadherin, N-cadherin, Snail, GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Cell lines and cell culture

Human cervical cancer cells (Hela) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in high-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell viability assay

Briefly, Hela cells were seeded in 96-well plates in normal medium at a density of $5\sim6 \times 10^3$ cells/100 μL/well and allowed to attach overnight. Cervical tumor cells were directly treated with AG490 (15-100 μmol/L) and axitinib (0.1-10 μmol/L) or in all possible combinations to yield desired concentrations of compounds continuously for 48 h. Cell viability was determined using XTT (Sigma, USA) assay as previously described [24]. All experiments were independently repeated three times for each concentration.

Annexin V/propidium iodide staining assay

To detect synergism effects of AG490 and axitinib on cell apoptosis, we stained the treated cells with FITC-Annexin V apoptosis kit (BD Biosciences, USA). Briefly, normal cultured Hela cells were directly exposed to the indicated concentrations of compounds of interest for consecutive 48 h. Cells were fixed and stained according to the manufacturer's instructions. The results were collected and analyzed by flow cytometry (FACSCalibur, BD Science, USA).

Flow cytometry analysis

To additionally evaluate cell cycle distribution, 2 10^6 cells were starved and treated with indicated concentration of AG490, axitinib and in combination with basic medium for 48 hours accordingly, harvested and washed in ice-cold PBS, then fixed in 70% ice-cold ethanol overnight. The cell pellets were resuspended in a 500 μL 50 mg/ml propidium iodide (PI)/RNase staining buffer (Sigma, USA) and incubated for 30 minutes at room temperature. Cell cycle distributions were determined by the analysis of nuclear DNA content using FACSscan cell analyzer (BD Biosciences, USA) equipped with Cellquest software (BD Biosciences, USA). Two independent experiments with triplicate were performed

Migration assay

To determine the effect of axitinib, AG490, or in combination on cell motility *in vitro*, Hela cells were allowed to grow to 90% confluence in six-well plates. Twenty-four hours after planting, cells were incubated with 10 μg/mL mitomycin C for 2 hours to inactivate cell proliferation. After that, a sterile 100-μL pipette tips were used to longitudinally scratch a constant-diameter stripe in the confluent monolayer to simulate a wound. Cells were washed with PBS, then incubated with AG490 and axitinib alone or in combination for 14 to 16 hours. Images of the cells were fixed and photographed. The migrated cells were counted manually, and the percentage of inhibition was expressed using untreated wells at 100%. Three independent experiments were performed.

Transwell migration assay

Cell migration was assayed in Boyden chambers (BD Biosciences, USA). A number of $5\sim7 \times 10^4$ cells/ treatment in 100 μL 10% FBS medium were pretreated with indicated concentrations of AG490 , axitinib or in combination for 1 h and then added to the upper chamber, while 500 μL medium were added to the lower chamber. After 48 h of migration at 37°C, the cells on the top surface of the membrane (nonmigrated cells) were scraped with a cotton swab, and cells on the underside of the membrane (invasive cells) were fixed with paraformaldehyde, stained with 1% crystal violet. Photographs of four random fields were taken using Olympus inverted microscope, and the cells were quantified by manual counting based on untreated control wells. Similar patterns of the inhibition effects were observed in three independent experiments.

Western blotting analysis

To determine changes in indicated proteins, cells (2×10^6) were plated in 6-well plates in 3 mL medium containing 10% FBS, incubated for 24 hours, and then treated with the compounds of interest as described earlier. Total cell lysates were prepared in RIPA buffer (20 mmol/L Tris, 2.5 mmol/L EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mmol/L $Na_4P_2O_7$, 40 mmol/L NaF, and 1 mmol/L PMSF) containing 1% phosphatase inhibitor cocktail (Sigma, USA) and subjected to Western blot analysis. The membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin in 0.1% Tween 20 in Tris-buffered saline and probed with specific antibodies overnight at 4°C, followed by exposure to the appropriated horseradish peroxidase-linked IgG antibody (Cell Signaling Technology, USA) and detected by an ECL system, as described previously [25]. Protein concentration was determined using bicinchoninic acid assay and equalized before loading. Relative optical density of blotting bands was qualified by Image J software (NIH; Bethesda, MD, USA).

Statistics

All values are presented as mean S.D. The Student's *t* test was used to evaluate the statistical significance of the results between groups. Differences between values were considered significant when *p* d"0.05.

Results

Effect of axitinib and AG490 on cell proliferation of cervical cancer in vitro

To systematically address the inhibitory activity of combining therapy on tumor growth, in our study, the cytotoxicity effects of single agent axitinib, AG490 and in any possible combinations were first evaluated in cervical cancer Hela cells by XTT assay. Following 48 hours exposure to compounds of interest, Hela cell line displayed differential sensitivity. As shown in **Figure 1B**, axitinib or AG490 alone demonstrated moderate cell viability suppression on Hela cells in *vitro*, with an IC_{50} value about 10 μmol/L and 100 μmol/L (**Figure 1A and B**). However, simultaneous treatment of axitinib (1 μmol/L) and AG490 (50 μmol/L) synergistically achieved a half maximum inhibition on cell proliferation. Both effects were dose-dependent.

Effect of axitinib and AG490 on apoptosis induction and cell cycle arrest of cervical cancer in vitro

We determined the optimal combined dose of axitinib and AG490 based on cytotoxicity test, with the aim of determining a dose that has minimal toxicity but retains good efficacy. Therefore, in all combinations, the dose of axitinib (1 μmol/L) and AG490 (50 μmol/L) were chosen for further experimentation. First, apoptosis was evaluated to determine whether the synergistic interactions between axitinib and AG490 led to induction of programmed cell death. Apoptosis, in response to axitinib and AG490 alone or in combination, was

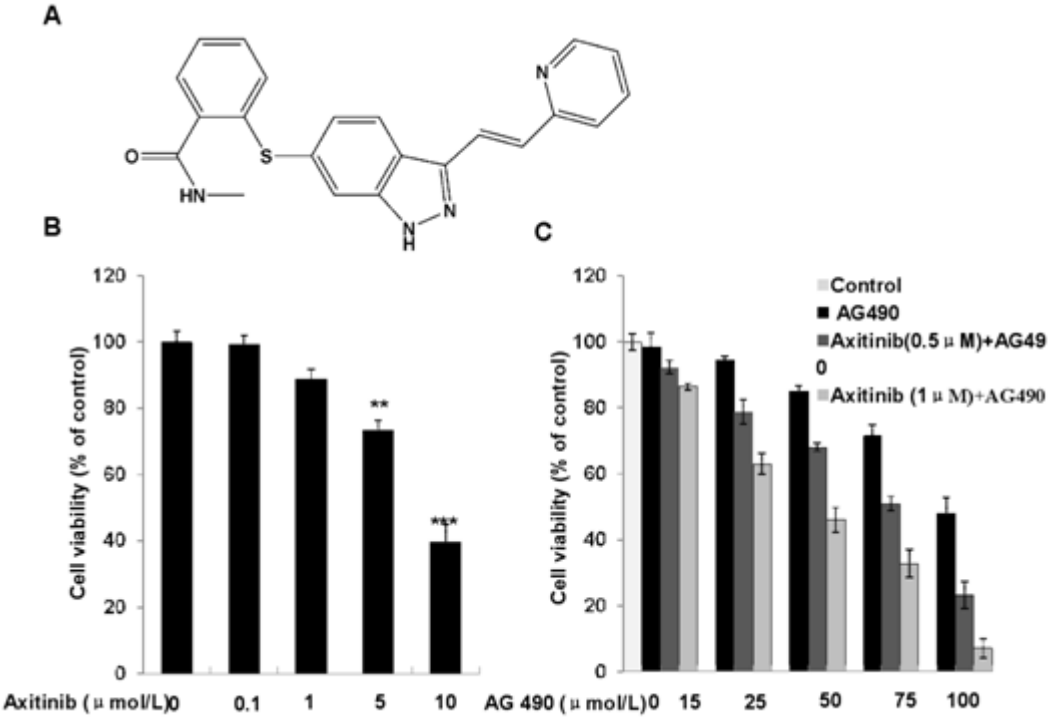

Figure 1. Effect of axitinib and AG490 on cell proliferation of cervical cancer *in vitro*. **A:** the chemical structure of axitinib. **B:** axitinib inhibited human cervical cancer cells proliferation in a dose-dependent manner. **C:** growth inhibitory effects of treatment with AG490 (15, 25, 50, 75, 100 μmol/L) in combination with axitinib(0.5, 1 μmol/L). Human Hela cancer cells (5 to 6 × 10^3 cells per well) were treated with different concentrations of AG490, axitinib or in combination for 48 hours as described in the Materials and Methods. Cell viability was quantified by XTT assay. Similar results were obtained in three independent experiments. Columns = mean, bars = standard deviation, ** p <0.01, *** p <0.001 vs. control

measured by Annexin V/propidium iodide staining assay. Our results showed that there was a slight increase in the proportion of apoptotic cells from 3.63% to 11.01% in Hela cells after 48 hours exposure to AG490 (50 μmol/L) and axitinib (1 μmol/L), however, this increase was not significant when compared the appropriate single agent controls (5.65% for AG490 and 7.09% for axitinib treatment alone) as shown in **Figure 2A**.

Accumulating reports demonstrated that some tyrosine kinase inhibitors have shown to induce either G0/G1 or S phase arrest, whereas, AG490 is known to induce G0/G1 cell cycle arrest [26]. We further assessed the synergistic effect of axitinib, AG490 and in combination on cell cycle distribution by flow cytometric analysis in cervical cancer cells. As depicted in figure 2B, Hela cells underwent a slight G0-G1 phase arrest in response to AG490 (50.40%, 50 μmol/L) and axitinib (56.48%, 1 μmol/L) alone, however, the proportion of cells in the G0/G1 phase was remarkably increased from 39.35% to 77.82% following exposure to these two agents compared to the untreated cells (**Figure 2B**).

Effect of axitinib and AG490 on chemotactic motility of cervical cancer in vitro

To assess the anti-metastatic function of axitinib/AG490 combination *in vitro*, we further investigated those inhibitory effects on chemotactic motility in Hela cells, implicated in key steps in tumor metastasis by wound-healing migration assay and the Boyden chamber invasion assay, respectively. The results showed that significantly enhanced inhibitory effects on both the decreased number of migrated cell into the scratched gap (**Figure 3**) and dramatically reduced cell invasion (**Figure 4**) were observed, suggesting that the combination therapy of AG490 with axitinib nearly completely abrogated the migration ability of Hela cells *in vitro*.

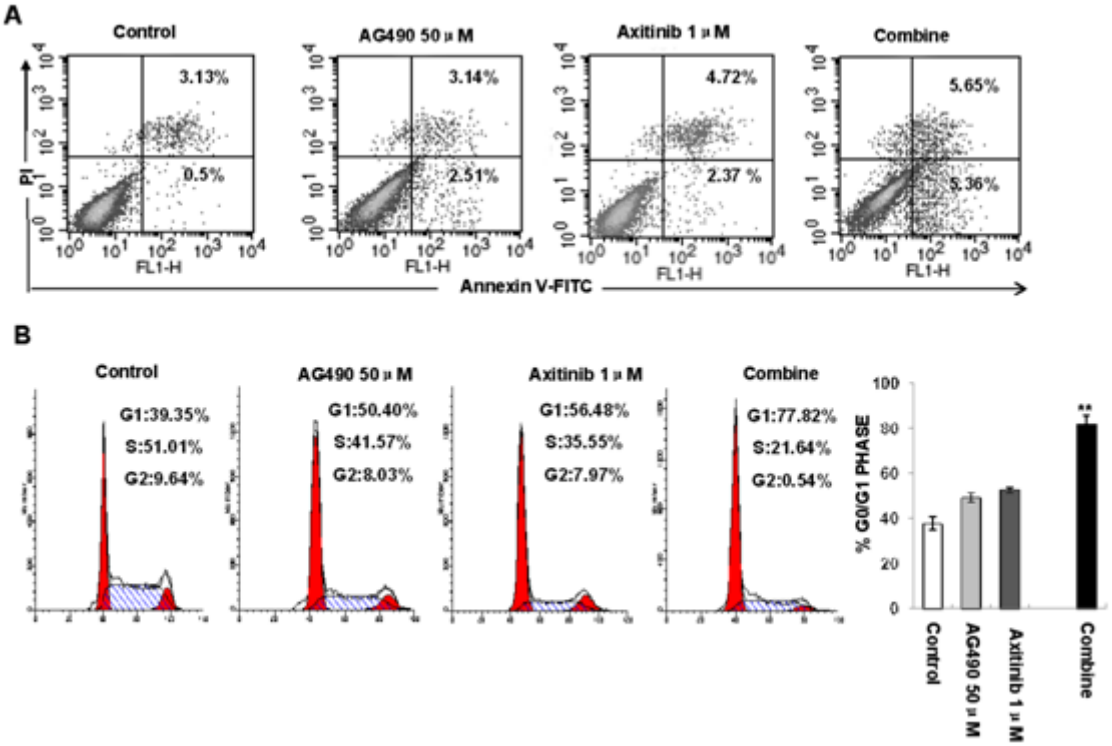

Figure 2. Effect of axitinib and AG490 on apoptosis induction and cell cycle arrest of cervical cancer *in vitro*. **A:** axitinib, AG490 or in combination has little effect on apoptosis in human cervical cancer Hela cells. **B:** the combined treatment with axitinib and AG490 inhibited the progression of cell cycle through G1 phase arrest in Hela cells. Cancer cells were treated with axitinib (1μmol/L) and AG490 (50 μmol/L) for 48 hour, fixed and stained with PI (25 μg/ml), then subjected to flow cytometry analysis (cell count versus PI/DNA content).

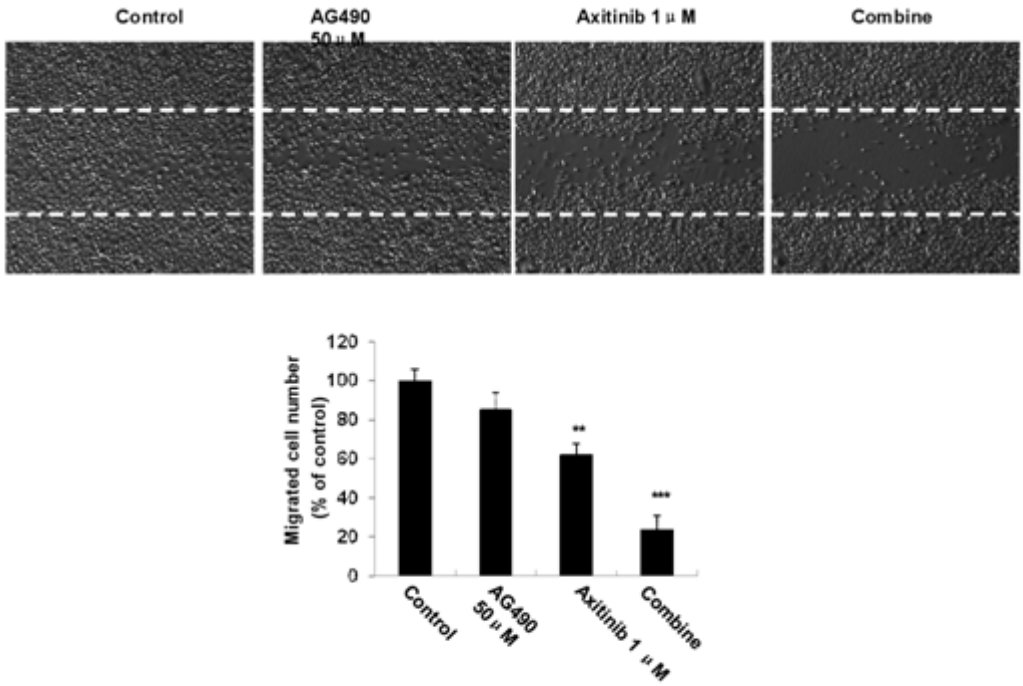

Figure 3. The combined treatment with axitinib and AG490 inhibited migration of cervical cancer. Cells were grown into full confluence in six-well plates and treated with indicated concentrations of axitinib, AG490 or in combination as described in the Materials and Methods. The migrated cells were quantified by manual counting. All of the results shown are representative of three independent experiments. Columns = mean, bars = standard deviation, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control

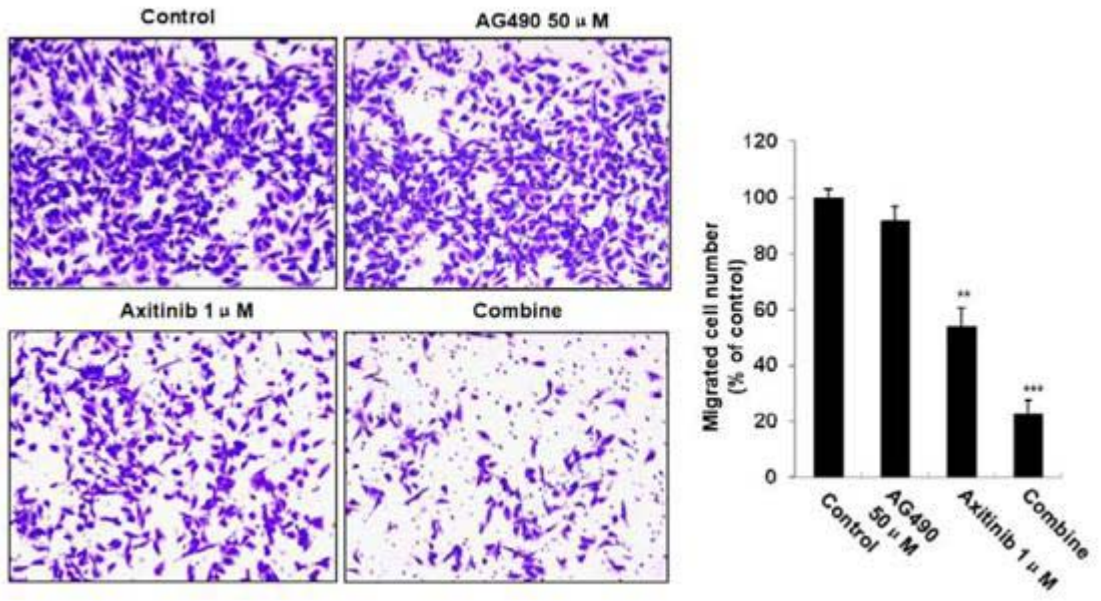

Figure 4. The combined treatment with axitinib and AG490 inhibited Transwell migration of cervical cancer. Cells were seeded in the upper chamber of a Transwell and treated with axitinib, AG490 or in combination as described in the Materials and Methods. After 48 hours, the number of Hela cells that migrated through the membrane was quantified. The drug combination treatment showed remarkable cell mobility inhibition as compared with the counterparts. All of the results shown are representative of three independent experiments. Columns = mean, bars = standard deviation, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control

Molecular basis of axitinib and AG490 in tumor growth arrest and metastasis inhibition

In order to delineate the molecular basis of axitinib and AG490 in anti-tumor growth and anti-metastatic procession, we analyzed the key signaling molecules and pathways mediated by axitinib and AG490 in Hela cells using western blots. We first examined whether axitinib and/or AG490 could effectively inhibit its target receptors, VEGFR2. **Figure 5A** shows that the level of VEGFR2 phosphorylation at Tyr 1175 site was almost fully inhibited in response to AG490 (50 μmol/L) and axitinib (1 μmol/L) co-treatment for 48 hours, but no significant change on the p-VEGFR2 was observed when cells were exposed to AG490 alone. In addition, treatment with axitinib also decreased the activity of p-VEGFR2, reduction in this protein was most marked when the drugs were used in combination, indicating that inactivation of p-VEGFR2 was a consistent effect of the combination leading to synergy. We similarly verified the effects of combined therapy of AG490 and axitinib on activity of the downstream effector, JAK2/STAT3 in Hela cell line. As expected, AG490 and axitinib synergistically blocked the phosphorylation of JAK2 ($Tyr^{1007/1008}$) and resulted in a rapid deactivation of STAT3 (Tyr^{705}).

Having established a strong link between JAK2/STAT3 and tumor metastasis-associated EMT, we further investigated the effect of axitinib and AG490 combination on the expressions of protein Snail, N-cadherin and E-cadherin, commonly used as markers of the epithelial-mesenchymal phenotype in EMT [27]. As seen in fig. 5B, treatment with the two agents could significantly reduce the expression of Snail while blocking N-cadherin and increasing E-cadherin. Together, our results indicate that simultaneous targeting of VEGFR2 and JAK2 by combining therapy of axitinib and AG490 effectively led to growth arrest and metastasis suppression in human cervical cancer, a biological effect that appears to be via VEGFR2/JAK2/STAT3 mediated epithelial-mesenchymal transition suppression.

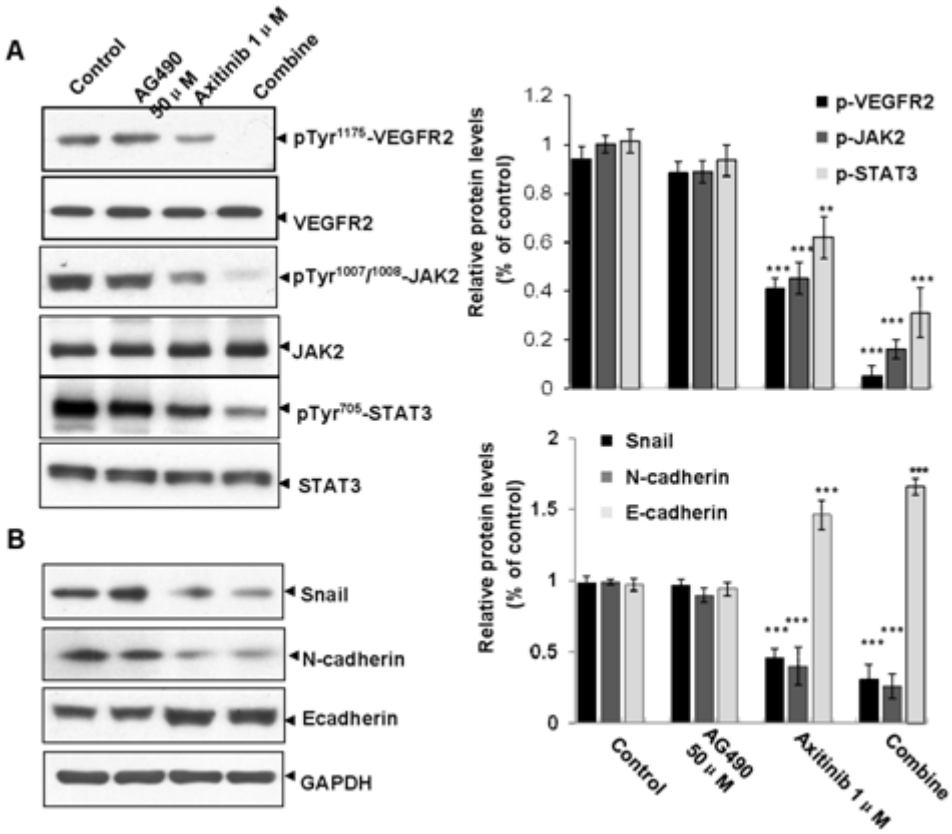

Figure 5. Molecular basis of axitinib and AG490 in tumor growth arrest and metastasis inhibition. **A:** axitinib/AG490 combined treatment significantly inhibited the activation of vascular endothelial growth factor receptor 2 (VEGFR2) and downstream signaling cascade, including phosphorylation of JAK2 at $Tyr^{1007/1008}$ and STAT3 at Tyr^{705}. Proteins from different treatments were analyzed by western blotting assay. **B:** axitinib and AG490 synergistically inhibited the expression of N-cadherin and increased the levels of E-cadherin through down-regulating Snail protein expression. GAPDH were used as internal controls. Columns = mean, bars = standard deviation, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control

Discussion

Therapeutic resistance has been the major mechanism underlying tumor recurrence and metastasis in cervical cancer. Recent studies have showed that EMT not only confers tumor cells with a distinct advantage for metastatic dissemination, but also it provides tumor cells for proliferation and chemotherapy resistance [3]. Thus, inhibiting this process using single or multiple agents, remains a field of intensive research. Our study clearly demonstrated, for the first time, that the combination therapy of VEGF-receptor blocker with JAK2-specific inhibitor, axitinib and AG490, is expected to effectively block the nodes of intracellular signaling cascade related to the growth and EMT-mediated tumor metastasis in cervical tumor cells *in vitro*, holds promise for an efficacious treatment of these advanced patients.

Previously reports showed that AG490, a tyrosine kinase inhibitor with activity against JAK2, blocked cell growth and inhibited tumor cell motility in *vitro* and in *vivo* [28], which resulted in an encouraging but still limited efficacy in the monotherapy setting [15]. In the present study, we further confirmed that AG490 alone dose-dependently inhibited cell proliferation in the Hela cell line, with the relatively high IC_{50} value at 100 μmol/L, which possibly limited its development and application in clinical trials. In additional, we found that axitinb also typically exhibited tumor cytostatic activity in cervical cancer cells, with the half maximal inhibitory concentration (IC_{50}) of about 10 μmol/L. Our results appeared to be in a similar range with preclinical studies reported in the literatures for proliferation inhibition in multiple cancer cell lines, including neuroblastoma [29], glioblastoma [30], prostate [16], breast [17]and non-small cell lung cancer [17]. The identification of optimal dosing regiments and schedules is critical to guide the design of clinical study evaluation of cancer therapeutics, especially when therapies are combined. In our experiments, we estimated the difference of anti-proliferative effects of combining axitinib and AG490 treatment using different schedules against Hela cells (data not shown). Interestingly, strong synergistic antitumor activities were observed, when simultaneous and continuous treatment with the two agents was done in *vitro* cell culture condition. Therapy with different doses of axitinib (1 μmol/L) and AG490 (50 μmol/L) significantly resulted in a 40% decrease of cervical cancer cells viability as well as a 27.42% enhancement accumulation of cells at G0-G1 phase without potently effect on apoptosis induction (10.46%) relative to AG490 monotherapy in *vitro*. When compared to the untreated cells, the inhibition effect was further intensified. However, AG490 alone, at the concentration of 50 μmol/L, was insufficient to provide a beneficial therapeutic effects in Hela cells. Our findings suggested that combination therapy is significantly superior to either single agent therapy.

Tumor metastasis is a complex multi-step process, which may allow cancer cells to detach from their lattice and to become migratory and invasive. In this study, we found that axitinib was able to decrease the cell motility of cervical cancer cells in *vitro*. When estimating the inhibition effect of simultaneous exposure to AG490 and axitinib at the well tolerated doses of 50 μmol/L and 1 mol/L, we found that the drug combination showed remarkable cell mobility inhibition as compared with the counterparts (**Figures 3 and 4**). Strongly consistent with previous finding, no toxic deaths occurred. Above results first provided the *in vitro* evidences that axitinib/AG490 combined treatment potentiated proliferation inhibition of cervical cancer cells *in vitro* through blocking cell-cycle progression and impairing cell mobility, implying that the enhanced antitumor effects of axitinib and AG490 are mediated primarily through anti-proliferative and anti-metastatic rather than pro-apoptotic properties.

As a validated target, constitutively activated VEGFR2 has been shown to be highly expressed in human cervical cancer [31]. Both VEGF receptor tyrosine kinases and their downstream targets are implicated to be crucial for tumor growth, angiogenesis and metastasis [32, 33]. Currently, axitinib, is a proven targeting VEGFR1, 2, 3 inhibitor and has great selectivity against VEGFR2. Our present investigation showed that simultaneous treatment with axitinib and AG490 could clearly block the phosphorylation of VEGFR2 (Tyr^{1175}) in Hela cells, which might lead to the inhibition of a number of downstream signaling cascades. STAT3 is principally activated by non-receptor tyrosine kinase JAK2 [34] , and c-Src family kinase has also been involved in STAT3 phosphorylation [35]. Our results showed that both phosphorylation of JAK2 ($Tyr^{1007/1008}$) and STAT3 (Tyr^{705}) was completely inhibited(Fig.5A), indicating that the direct effects of combining VEGFR inhibitors and JAK2 inhibitors mediated- synergism on tumor growth and metastasis might be partly explained through inhibiting the VEGFR2/JAK2/STAT3 signaling cascade. In addition, previous reports have shown a

close association between STAT3 signaling and the sustention of EMT-associated metastasis [36, 37]. When noted, above inhibitory action was accompanied by down-regulating Snail in parallel with significant reduction expressions of N-cadherin and obvious enhancement levels of E-cadherin. Loss of E-cadherin, a cell-adhersion protein, was considered as one of the key hallmarks of EMT, which is regulated by multiple transcriptional factors including Snail, Slug, or Twist. One recent publication showed that Snail might participate in the collective migration of cancer cells, possibly by endowing migrating cancer cells with resistance against cell death, however, inhibition Snail could impair the propensity for infiltration or distant metastasis in vivo as evidenced by decreased tumor-sphere formation [38]. These data highlight the potential significance of VEGFR2/JAK2/STAT3 cascade in the acquisition of EMT-associated phenotypes of cervical cancer, which may be important for cancer progression and distant metastasis, and similar results have reported in other cancer types [37, 39]. Here, our finding supported previous hypothesis that axitinib and AG490 combination was highly synergistic on anti tumor effect in vitro, through interfering VEGFR2/JAK2/STAT3 signaling-mediated growth arrest and EMT inhibition.

In conclusion, our results provide a rational experiment basis for the clinical development of a therapeutic approach based on this particular combination in cervical cancer treatment. However, detail mechanisms responsible for the synergism remain to be elucidated.

Acknowledgments

Rong-rong Zhang was supported by grant (No: 11yj013) from the Xinhua Hospital Foundation. Rong-Rong Zhang and Hong Wang contributed equally to this work, and they are the co-first authors. The authors declare no potential conflict of interests.

References

1. Jemal A, Bray F, MM Center, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69-90.
2. Pectasides D, Kamposioras K, Papaxoinis G, Pectasides E. Chemotherapy for recurrent cervical cancer. Cancer Treat Rev. 2008; 34:603-13.
3. Sirica AE, Campbell DJ, Dumur CI. Cancer-associated fibroblasts in intrahepatic cholangiocarcinoma. Curr Opin Gastroenterol. 2011; 27:276-84.
4. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol. 2006; 172:973-81.
5. Lee MY, Chou CY, Tang MJ, Shen MR. Epithelial-mesenchymal transition in cervical cancer: correlation with tumor progression, epidermal growth factor receptor overexpression, and snail up-regulation. Clin Cancer Res. 2008; 14:4743-50.
6. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol. 2006; 7:131-42.
7. Quintas-Cardama AK, Vaddi P L, Manshouri T, Li J, Scherle PA, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. Blood. 2010; 115:3109-17.
8. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res. 2002; 8:945-54.
9. Gamero AM, Young HA, Wiltrout RH. Inactivation of Stat3 in tumor cells: releasing a brake on immune responses against cancer? Cancer Cell. 2004; 5:111-2.
10. Lee HJ, Seo NJ, Jeong SJ, Park Y, Jung DB, Koh W, et al. Oral administration of penta-O-galloyl-beta-D-glucose suppresses triple-negative breast cancer xenograft growth and metastasis in strong association with JAK1-STAT3 inhibition. Carcinogenesis. 2011; 32:804-11.
11. Dong Y, Lu B, Zhang X, Zhang J, Lai L, Li D, et al. Cucurbitacin E, a tetracyclic triterpenes compound from Chinese medicine, inhibits tumor angiogenesis through VEGFR2-mediated Jak2-STAT3 signaling pathway. Carcinogenesis. 2010; 31:2097-104.
12. Zhang X, Song Y, Wu Y, Dong Y, Lai L, Zhang J, et al. Indirubin inhibits tumor growth by antitumor angiogenesis via blocking VEGFR2-mediated JAK/STAT3 signaling in endothelial cell. Int J Cancer. 2011; 129:2502-11.
13. Yadav A, Kumar B, Datta J, Teknos TN, Kumar P. IL-6 Promotes Head and Neck Tumor Metastasis by Inducing Epithelial-Mesenchymal Transition via the JAK-STAT3-SNAIL Signaling Pathway. Mol Cancer Res. 2011; 9:1658-1667.
14. Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N, et al. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene. 2009; 28:2940-7.
15. Kapuria V, Levitzki A, Bornmann WG, Maxwell D,

Priebe W, Sorenson RJ, et al. A novel small molecule deubiquitinase inhibitor blocks Jak2 signaling through Jak2 ubiquitination. Cell Signal. 2011; 23:2076-85.
16. Canu B, Fioravanti A, Orlandi P, Di Desidero T, Ali G, Fontanini G, et al. Irinotecan synergistically enhances the antiproliferative and proapoptotic effects of axitinib in vitro and improves its anticancer activity in vivo. Neoplasia. 2011; 13:217-29.
17. Hu-Lowe DD, Zou HY, Grazzini ML, Hallin ME, Wickman GR, Amundson K, et al. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. Clin Cancer Res. 2008; 14: 7272-83.
18. Rixe O, Bukowski RM, Michaelson MD, Wilding G, Hudes GR, Bolte O, et al. Axitinib treatment in patients with cytokine-refractory metastatic renal-cell cancer: a phase II study. Lancet Oncol. 2007; 8: 975-84.
19. Cohen EE, Rosen LS, Vokes EE, Kies MS, Forastiere AA, Worden FP, et al. Axitinib is an active treatment for all histologic subtypes of advanced thyroid cancer: results from a phase II study. J Clin Oncol. 2008; 26:4708-13.
20. Schiller JH, Larson T, Ou SH, Limentani S, Sandler A, Vokes E, et al. Efficacy and safety of axitinib in patients with advanced non-small-cell lung cancer: results from a phase II study. J Clin Oncol. 2009; 27: 3836-41.
21. Sharma SV, Abhyankar RE, Burgess J, Infante RC, Trowbridge J, Tarazi, et al. A phase I study of axitinib (AG-013736) in combination with bevacizumab plus chemotherapy or chemotherapy alone in patients with metastatic colorectal cancer and other solid tumors. Ann Oncol. 2010; 21:297-304.
22. Fruehauf J, Lutzky J, McDermott D, Brown CK, Meric JB, Rosbrook B, et al. Multicenter, Phase II Study of Axitinib, a Selective Second-Generation Inhibitor of Vascular Endothelial Growth Factor Receptors 1, 2, and 3, in Patients with Metastatic Melanoma. Clin Cancer Res. 2011; 17:7462-9.
23. Spano JP, Chodkiewicz C, Maurel J, Wong R, Wasan H, Barone C, et al. Efficacy of gemcitabine plus axitinib compared with gemcitabine alone in patients with advanced pancreatic cancer: an open-label randomised phase II study. Lancet. 2008; 371:2101-8.
24. Kanthamneni N, Chaudhary A, Wang J, Prabhu S. Nanoparticulate delivery of novel drug combination regimens for the chemoprevention of colon cancer. Int J Oncol. 2010; 37:177-85.
25. Pang X, Yi Z, Zhang X, Sung B, Qu W, Lian X, et al. Acetyl-11-keto-beta-boswellic acid inhibits prostate tumor growth by suppressing vascular endothelial growth factor receptor 2-mediated angiogenesis. Cancer Res. 2009; 69:5893-900.
26. Fuke H, Shiraki K, Sugimoto K, Tanaka J, Beppu T, Yoneda K, et al. Jak inhibitor induces S phase cell-cycle arrest and augments TRAIL-induced apoptosis in human hepatocellular carcinoma cells. Biochem Biophys Res Commun. 2007; 363:738-44.
27. Li H, Wang H, Wang F, Gu Q, Xu X. Snail involves in the transforming growth factor beta1-mediated epithelial-mesenchymal transition of retinal pigment epithelial cells. PLoS One. 2011; 6:e23322.
28. Huang C, Yang G, Jiang T, Huang K, Cao J, Qiu Z. Effects of IL-6 and AG490 on regulation of Stat3 signaling pathway and invasion of human pancreatic cancer cells in vitro. J Exp Clin Cancer Res. 2010; 29:51.
29. Rossler J, Monnet Y, Farace F, Opolon P, Daudigeos-Dubus E, Bourredjem A, et al. The selective VEGFR1-3 inhibitor axitinib (AG-013736) shows antitumor activity in human neuroblastoma xenografts. Int J Cancer. 2011; 128:2748-58.
30. Ma J, Waxman DJ. Modulation of the antitumor activity of metronomic cyclophosphamide by the angiogenesis inhibitor axitinib. Mol Cancer Ther. 2008; 7:79-89.
31. Longatto-Filho A, Pinheiro C, Martinho O, Moreira MA, Ribeiro LF, Queiroz GS, et al. Molecular characterization of EGFR, PDGFRA and VEGFR2 in cervical adenosquamous carcinoma. BMC Cancer. 2009; 9:212.
32. Pang X, Wu Y, Lu B, Chen J, Wang J, Yi Z, et al. (-)-Gossypol suppresses the growth of human prostate cancer xenografts via modulating VEGF signaling-mediated angiogenesis. Mol Cancer Ther. 2011; 10: 795-805.
33. Pang X, Yi Z, Zhang J, Lu B, Sung B, Qu W, et al. Celastrol suppresses angiogenesis-mediated tumor growth through inhibition of AKT/mammalian target of rapamycin pathway. Cancer Res. 2010; 70:1951-9.
34. Ren Z, Schaefer TS. ErbB-2 activates Stat3 alpha in a Src- and JAK2-dependent manner. J Biol Chem. 2002; 277:38486-93.
35. Schreiner SJ, Schiavone AP, Smithgall TE. Activation of STAT3 by the Src family kinase Hck requires a functional SH3 domain. J Biol Chem. 2002; 277: 45680-7.
36. Xu Z, Jiang Y, Steed H, Davidge S, Fu Y. TGFbeta and EGF synergistically induce a more invasive phenotype

of epithelial ovarian cancer cells. Biochem Biophys Res Commun. 2010; 401:376-81.

37. Colomiere M, Ward AC, Riley C, Trenerry MK, Cameron-Smith D, Findlay J, et al. Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial-mesenchymal transition in ovarian carcinomas. Br J Cancer. 2009; 100:134-44.
38. Dang H, Ding W, Emerson D, Rountree CB. Snail1 induces epithelial-to-mesenchymal transition and tumor initiating stem cell characteristics. BMC Cancer. 2011; 11:396.
39. Zhao S, Venkatasubbarao K, Lazor JW, Sperry J, Jin C, Cao L, et al. Inhibition of STAT3 Tyr705 phosphorylation by Smad4 suppresses transforming growth factor beta-mediated invasion and metastasis in pancreatic cancer cells. Cancer Res. 2008; 68:4221-8.