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Inhibition of janus kinase 2 by compound AG490 suppresses the proliferation of MDA-MB-231 cells via up-regulating SARI (suppressor of AP-1, regulated by IFN)

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ABSTRACT

Objective(s): The Janus kinase-signal transducers and activators of transcription signaling pathway (JAK/STAT pathway) play an important role in proliferation of breast cancer cells. Previous data showed that inhibition of STAT3 suppresses the growth of breast cancer cells, but the associated mechanisms are not well understood. This study aims to investigate the effect and associated mechanisms of JAK/STAT pathway inhibitor AG490 on proliferation and suppression of breast cancer cells.

Materials and Methods: CCK-8 assay and trypan blue exclusion assay were used to investigate the cytotoxicity of AG490 to MDA-MB-231 cells. Real-time PCR was used to detect the mRNA level of SARI (suppressor of AP-1, regulated by IFN). Western blot was used to analyze the protein levels of SARI, phospho-STAT3 and total STAT3. Luciferase reporter assay was adopted to explore the mechanism of SARI mRNA upregulation.

Results: AG490 suppressed the proliferation of MDA-MB-231 cells in a dose-dependent manner. AG490 significantly up-regulated the mRNA and protein levels of SARI in MDA-MB-231 cells. Knockdown of SARI obviously attenuated AG490-induced growth suppression effect in MDA-MB-231 cells. Furthermore, AG490 dramatically enhanced the transcription activity of SARI promoter. But the transcription activity of truncated SARI promoter, which does not contain STAT3 binding site, cannot be activated by AG490 treatment.

Conclusion: We demonstrate in this study that AG490 suppresses the proliferation of MDA-MB-231 cells through transcriptional activation of SARI.

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Introduction

Breast cancer is one of the leading causes of mortality in women (1). Currently, the efficacy of traditional chemotherapy for breast cancer is often unsatisfactory (2). Therefore, it is of great priority to develop novel molecular targeted compounds. Recent studies have shown that the inhibitors of the janus kinase-signal transducers and activators of transcription signaling pathway (JAK/STAT pathway) exhibit promising antitumor activity (3).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which relays signal from cell surface receptors to the nucleus (3). Persistent activation of STAT3 is frequently found in majority of human cancers including breast cancer (4). Aberrant STAT3 activation in tumor cells is associated with cell proliferation, cell survival, invasion, angiogenesis, and metastasis (5). Conversely, targeting STAT3 activation inhibits tumor growth and metastasis both in vitro and in vivo without affecting normal cells, thus suggesting that STAT3 could be a valid molecular target for cancer therapy (4).

Several compounds have been identified that can block STAT3 activation in a variety of preclinical models both in vitro and in vivo (6). AG490 is a kind of derivative of benzylidene malononitrile which decreases tyrosine phosphorylation (7). AG490 inhibits the activity of janus kinase (JAK) 2 specifically and is used as an anticancer agent in clinic (8). AG490 can effectively suppress the proliferation of different cancer cells including human breast cancer cells (9-11). AG490 is also effective in various models of autoimmune and inflamematory diseases, and type 1 diabetes. AG490 exhibits

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favorable activity on STAT3 inhibition (12). Administration of AG490 reduced levels of activated STAT3, resulting in suppression of invasion and metastasis of human breast cancer cells (13). Combination therapy with AG490 and doxorubicin or AG490 and cardiotoxin III also induced greater anti-tumor effects in breast cancer cells (14-15). However, the cellular molecular mechanisms involved in the AG490-mediated growth suppression in breast cancer remain elusive.

In the present study, we for the first time demonstrated AG490 significantly up-regulated the mRNA and protein levels of SARI (suppressor of AP-1, regulated by IFN) in MDA-MB-231 cells via STAT3 inhibition-mediated transcription activation. Moreover, knockdown of SARI attenuated AG490-induced suppression effect on MDA-MB-231 cell growth. This study may provide novel insights into the STAT3-targeted cancer therapeutics.

Materials and Methods

Cell culture and reagents

Human breast cancer cell line MDA-MB-231 was from American Type Culture Collection (ATCC). These cells were cultured in high-glucose DMEM with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). These cell culture reagents were purchased from Hyclone (Waltham, MA, USA). AG490 was purchased from Sigma-Aldrich (Louis, MO, USA), and dissolved in DMSO at 50 mM as stock solution.

Cell viability assay

Cell viability assay was detected by Cell Counting Kit-8 (CCK-8) from Dojindo (Shanghai, China). Cells were seeded in triplicate manner in 96-well plates and incubated in different treatment groups for 48 hr. Then they were detected for optical density (OD) value at 450 nm according to the manufacturer's protocol.

Trypan blue exclusion assay

The treated MDA-MB-231 cells were adequately suspended in 0.4% (w/v) trypan blue solution. Then the cells were counted under an optical microscope. The cells failing to exclude the dye (presented blue) were defined as dead cells. The total death rate (%) = numbers of dead cells / (numbers of living cells + numbers of dead cells) ×100

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were treated with AG490 for 24 hr, then they were lysed with TRIzol agent from Invitrogen (Carlsbad, CA, USA). Total RNA was extracted and first-hand cDNA was obtained by M-MLV transcriptase from Invitrogen. Real-time PCR was performed to detect the SARI mRNA levels using SYBR from TAKARA (Shiga, Japan) in a standard protocol in a 25 μl volume according to the manufacturer's protocol.

Knockdown of SARI

siRNA to SARI or control siRNA was obtained from Dharmacon (Lafayette, CO, USA). In the corresponding experiments, the MDA-MB-231 cells were seeded onto 6-well plates and transfected with siRNA (100 pmol/well) using Lipofectamine 2000 (Carlsbad, CA, USA) according to the manufacturer's instructions.

Plasmid construction

SARI promoter region from -2000 to +100 and -1700 to +100 were cloned from MDA-MB-231 cell genomic DNA using PrimeSTAR HS DNA polymerase from TAKARA (Shiga, Japan). Then these two PCR products were inserted into pGL3-basic plasmid. Then the connection products were transformed to competent cells of DH5α. Recombinant plasmids were sequenced afterwards.

Luciferase reporter assay

MDA-MB-231 cells were seeded in 48-well plates and transfected with constructed luciferase reporter plasmids. After 36 hr, cells were lysed and luciferase activity was detected using Dual-Luciferase Reporter Assay System (Promega). The luciferase activities were calculated as the constructs' luciferase activity relative to the activity of the Renilla reniformis luciferase produced by the pRL-SV40 control vector.

Western blot

Preparation of the whole-cell lysates and the protocol of Western blot were mentioned previously (16). Antibodies of SARI, STAT3 and p-STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GAPDH antibody was purchased from Beyotime Biotechnology (Shanghai, China).

Statistical analysis

The data were presented as Mean±SD from triplicate experiments. Two-way t-test and ANOVA were used to analyze the variance in different treatment groups. A threshold of $P<0.05$ was defined as statistically significant.

Results

AG490 suppressed the proliferation of MDA-MB-231 cells in dose-dependent manner

We first evaluated the sensitivity of MDA-MB-231 cells to AG490 by examining the cell growth inhibition and the total cell death. As shown in Figure 1A and 1B, AG490 induced growth inhibition and cell death in a dose-dependent manner in MDA-MB-231 cells, which was consistent with previous study (14). The half maximal inhibitory concentration (IC_{50}) value of AG490 in MDA-MB-231 cells is 28.327 μM.

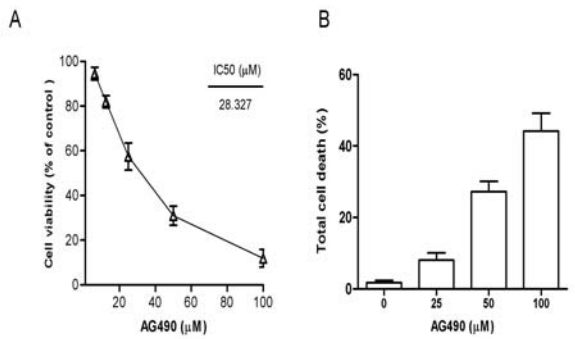

Figure 1. AG490 suppressed the proliferation of MDA-MB-231 cells in dose-dependent manner. A) MDA-MB-231 cells were treated with AG490 at indicated concentration for 48 hr, and then the cell viability was quantified by CCK-8 assay using DMSO group values as control. Data are mean±SD from three independent experiments. B) MDA-MB-231 cells were treated as above for 24 hr, and the total cell death was evaluated by trypan blue staining. Data are mean±SD from three independent experiments.

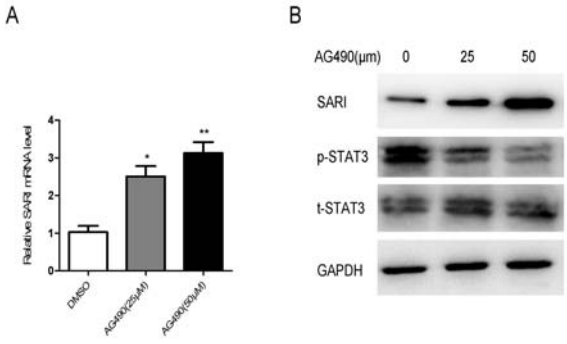

Figure 2. AG490 upregulated SARI (suppressor of AP-1, regulated by IFN) at both mRNA and protein levels. A) MDA-MB-231 cells were treated with AG490 at indicated concentration for 24 hr, then qPCR was performed to quantitate SARI mRNA, taking GAPDH as a control. Data were represented as mean±SD from three independent experiments. *: *P*<0.05, **: *P*<0.01. B) MDA-MB-231 cells were treated as described in A), then the level of SARI protein was analyzed by Western blotting, taking GAPDH as a loading control

AG490 upregulated SARI at both mRNA and protein levels

Next we investigated the effect of AG490 on the expression of anti-oncogene SARI. The results of real-time PCR (Figure 2A) showed that AG490 upregulated SARI mRNA in a dose-dependent manner. The results of Western blotting further showed that AG490 also upregulated SARI protein level and downregulated p-STAT3 level in a dose-dependent manner (Figure 2B).

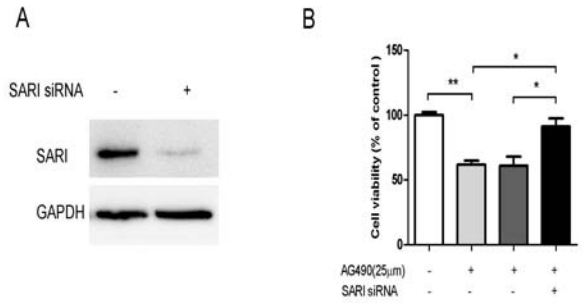

Figure 3. Knockdown of SARI (suppressor of AP-1, regulated by IFN) attenuated AG490-induced suppression effect on MDA-MB-231 cell growth. A) MDA-MB-231 cells were transfected with SARI siRNA or control siRNA for 36 hr, then SARI protein level was analyzed by Western blot. B) After transfection with SARI siRNA or control siRNA for 24 hr, the MDA-MB-231 cells were treated with 25 μM AG490 or vehicle DMSO for another 36 hr. Then the cell viability was analyzed by CCK-8 kit. Data were expressed as the mean ± SD from three independent experiments. *: *P*<0.05; **: *P*<0.01

Knockdown of SARI attenuated AG490-induced suppression effect on MDA-MB-231 cell growth

To test the role of SARI upregulation in AG490-treated cells, we knocked it down by siRNA in MDA-MB-231 cells (Figure 3A). Compared to AG490 treatment alone, knockdown of SARI significantly attenuated AG490-induced suppression effect on MDA-MB-231 cell growth (Figure 3B).

AG490 upregulated the promoter activity of SARI through suppressing STAT3

To figure out the mechanisms of AG490-mediated SARI upregulation, we tested the transcription activity of SARI upon AG490 treatment. Sequence analysis of the SARI promoter showed that there is one putative STAT3 binding site at -1797 to -1787 regions (Figure 4A). Hence, we cloned the promoter regions -2000 to +100 and -1700 to +100 of SARI gene into reporter vector pGL3-basic. Then MDA-MB-231 cells were transfected with report constructs for 24 hr and followed treatment with AG490 for another 36 hr. The results of luciferase report assay showed that AG490 increased luciferase activity in MDA-MB-231 cells transfected with promoter fragments -2.0 Kb, but not with -1.7 Kb (Figure 4B). These results demonstrated that AG490 upregulated the promoter activity of SARI through suppressing STAT3.

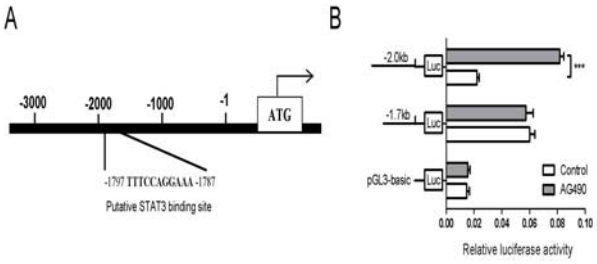

Figure 4. AG490 upregulated the promoter activity of SARI (suppressor of AP-1, regulated by IFN) through suppressing signal transducer and activator of transcription 3 (STAT3). A) SARI promoter region containing putative STAT3 binding sites. B) A schematic diagram of the SARI reporter fragments that were transfected into MDA-MB-231 cells and maintained in the absence or presence of 25 μM AG490. The right panel depicts the mean±SD of the relative luciferase activity measured in 3 different experiments. ***: *P*<0.001

Discussion

In the present study, we demonstrated that AG490 upregulates SARI by STAT3 suppression-mediated transcription activation in MDA-MB-231 cells. AG490 inhibited the activity of STAT3, and upregulated anti-oncogene SARI at both mRNA and protein level. Deletion of STAT3 binding site in SARI promoter eliminated the transcription activation effect of AG490 on SARI. Knockdown of SARI also attenuated AG490-induced suppression effect on MDA-MB-231 cell growth.

AG490 is a tyrosine kinase inhibitor specifically for JAK2 and associated JAK2-STAT3 pathway. AG490 blocked leukemic cell growth by inducing apoptosis (17). AG490 also reduced the DNA-binding activity of STAT3, resulting in growth suppression of myeloma cell (18). Consistent with previous studies (14), AG490 inhibits STAT3 activity and cell growth in MDA-MB-231 cells.

STAT3 is a transcription factor (19). Phosphorylated STAT3 can homodimerize or heterodimerize with STAT1 and transport into nucleus (20). STAT3 can regulate both oncogenes and tumor suppressor genes. STAT3 can directly up-regulate genes involving in cell survival (Bcl-xL, Mcl-1, c-FOS, HIF-1α and survivin), cell-cycle regulators (cyclin D1 and c-Myc) and inducers of angiogenesis (VEGF) (21). STAT3 also regulates genes for tumor suppressing (FOXO1 and FOXO3A) and genes that suppress cell proliferation and survival as well as tumor metastasis (p21WAF1/CIP1, p55α and p50α PI3K subunits) (21). Our study demonstrates that STAT3 also negatively regulates tumor suppressor gene SARI, which may contribute to MDA-MB-231 cell growth.

SARI also known as suppressor of AP-1,is regulated by IFN and has been implicated in cell-growth inhibition and apoptosis (22). SARI is down-regulated in various types of human cancers and plays an important role in tumor development (23). Loss of SARI facilitates EMT, leading to lung adenocarcinoma metastasis (24). SARI binds with c-Jun and suppresses DNA-binding activity of AP-1 complexes in breast cancer cells, which causes cell-cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity (25). SARI induction also inhibits AP-1–dependent gene expression (23). But to our knowledge, the regulation of SARI is not clear yet. Our results show that STAT3 represses the transcription of SARI through binding with its promoter. This may be in favor of tumor cell growth.

Conclusion

Our study for the first time demonstrates that SARI is negatively regulated by STAT3, and AG490 suppresses the proliferation of MDA-MB-231 cells at least partly via SARI up-regulation.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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