ORIGINAL RESEARCH

SLAMF1 Promotes Methotrexate Resistance via Activating Autophagy in Choriocarcinoma Cells

Dazun Shi
Yu Zhang
Yan Tian

Department of Gynecology and Obstetrics, Xiangya Hospital, Central South University, Changsha, Hunan Province, People's Republic of China

Objective: The acquisition of chemoresistance to methotrexate (MTX) still remains one of the major challenges for choriocarcinoma treatment. Herein, we aimed to evaluate the potential role of Signaling Lymphocytic Activation Molecule Family Member 1 (SLAMF1) as a possible regulator of chemoresistance to MTX in choriocarcinoma.

Material and Methods: MTX-resistant JEG3 and JAR sublines (JEG3/MTX, JAR/MTX) were used to study SLAMF1 function. CCK8 assay and soft agar assay were conducted to measure the cell viability and clonogenesis of choriocarcinoma cells, respectively; MDC incorporation assay was conducted for the quantification of intracellular autophagy; BrdU labeling was used to assess the proliferative potential of choriocarcinoma cells; SLAMF1 protein expression was analyzed by Western blotting.

Results: Upregulation of SLAMF1 expression was observed in MTX-resistant JEG3/MTX and JAR/MTX sublines compared to their parental JEG3 and JAR cell lines, respectively. Knockdown of SLAMF1 markedly attenuated cell viability and soft agar clonogenesis after incubation with MTX in JEG3/MTX and JAR/MTX cells. In contrast, constitutive expression of SLAMF1 rescued cell survival soft agar clonogenesis in JEG3 and JAR cells treated with MTX. Moreover, autophagy is apparently activated in MTX-resistant JEG3/MTX and JAR/MTX sublines compared to their parental cell lines. Autophagy inhibitor 3-methyladenine and bafilomycin A1 enhanced MTX-induced cytotoxicity in MTX-resistant JEG3 and JAR sublines. Further, SLAMF1 might activate autophagy-related mechanism to promote resistance to MTX in choriocarcinoma cells. Depletion of SLAMF1 suppressed autophagy and induced apoptosis in MTX-treated JEG3/MTX and JAR/MTX cells.

Conclusion: SLAMF1 might promote MTX resistance via activating protective autophagy in choriocarcinoma cell lines. Targeting SLAMF1 might be a useful therapeutic strategy to sensitize choriocarcinoma cells to MTX-based regimens.

Keywords: choriocarcinoma, methotrexate, drug resistance, SLAMF1, autophagy

Introduction

Choriocarcinoma is a very rare malignancy occurred in the placenta of pregnant women. The main biochemical marker, used in treatment and prognosis for choriocarcinoma is human chorionic gonadotropin (hCG). Choriocarcinoma is often characterized by its fast-growing and aggressive nature, which ultimately leads to patient death.[1] Methotrexate (MTX) has been successfully used for the treatment of choriocarcinoma for decades.[2] However, chemoresistance to MTX still represents a major challenge for choriocarcinoma treatment. A considerable fraction (20~30%) of low-risk choriocarcinoma may fail to achieve complete remission after MTX treatment.[3,4] Moreover, about 10~20%

Correspondence: Yan Tian
Department of Gynecology and Obstetrics, Xiangya Hospital, Central South University, Changsha 410008, People's Republic of China
Email tianyxycsu@163.com

patients with high-risk choriocarcinoma may render an incomplete remission to MTX-containing regimens.[5,6] Although chemoresistance to MTX in choriocarcinoma has been well recognized, its regulatory mechanisms are not fully understood. A number of molecular mechanisms responsible for MTX resistance are documented, including drug efflux via ATP-binding-cassette (ABC) transporters,[7,8] upregulation of dihydrofolate reductase (DHFR) gene,[9] inhibition of apoptosis,[10] involvement of interferon signaling,[11] and enrichment of cancer stem cell fraction.[12] Recently, our quantitative proteomics analysis identified multiple genes/pathways potentially associated with chemoresistance to MTX in JEG3 choriocarcinoma cells, which still awaits functional validation.[13]

Signaling Lymphocytic Activation Molecule Family Member 1 (SLAMF1, CD150) belongs to signaling lymphocyte activation molecule (SLAM) family of cell-surface receptors in hematopoietic cells.[14] In hematologic malignancies, SLAMF1 expression is mainly expressed in cutaneous T-cell lymphomas, Hodgkin's lymphoma, chronic lymphocytic leukemia, and multiple myeloma.[15–17] In chronic lymphocytic leukemia and Hodgkin's lymphoma, SLAMF1 plays an critical role in malignant cell fate decision and formation of tumor microenvironment.[18,19] Recently, SLAMF1 has been shown to exert a crucial role in autophagy in chronic lymphocytic leukemia cells.[20] However, the function of SLAMF1 in the regulation of MTX resistance still remains largely unknown. In our previous proteomics study, highly elevated expression of SLAMF1 was observed in MTX-resistant JEG3/MTX subline compared to parental JEG3,[13] which sparked our interest to investigate its function in the chemoresistance to MTX in choriocarcinoma.

Materials and Methods
Reagents and Cell Lines
Primary antibodies used in this study were provided by the following sources: SLAMF1 (PA5-96046), Invitrogen (Carlsbad, CA, USA); β-actin (#58169), LC3B (#2775), p62 (#8025), and cleaved Caspase-3 (#9664), Cell Signaling (Danvers, MA, USA). MTX, Monodansylcadaverine (MDC), Bafilomycin A1, 5-bromo-2′-deoxyuridine (BrdU), and 3-Methyladenine (3-MA) were provided by Sigma-Aldrich (St. Louis, MO, USA). Human choriocarcinoma cell lines JEG3

and JAR were provided by ATCC culture collection (Manassas, VA, USA). MTX-resistant JEG3/MTX and JAR/MTX sublines were established as we described previously.[13] The use of these choriocarcinoma cell lines in this study was approved by the institutional research ethics committee in Xiangya Hospital, Central South University. These cell lines were routinely grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Lentiviral plasmids expressing non-targeting scramble or shSLAMF1 were provided by Genecopoeia Inc. (Rockville, MD, USA). Lentiviral plasmids LV105 expressing empty vector or SLAMF1 open reading frames (ORFs) were also provided by Genecopoeia Inc. The production of recombined lentivirus was performed according to the manufacturer's instruction.[13]

Soft Agar Clonogenesis Assay
Soft agar clonogenesis assay was used to examine the clonogenesis potential of choriocarcinoma cells as we described previously.[21] After incubation with or without MTX for 10~12 days, the colonies (>50 cells) were counted.

Cell Viability Analysis
Cell viability was evaluated with CCK-8 assay as described previously.[13] Briefly, choriocarcinoma cells were seeded in a 96-well plate in triplicate (3×10^3 cells/well). Cell viability was measured at 48 hours after incubation with MTX. IC_{50} values were calculated in SPSS software as we described previously.[13]

BrdU Incorporation Assay
BrdU incorporation assay was conducted using Cell Proliferation Assay Kit (Cell Signaling, Boston, MA) as we described previously.[13] Briefly, choriocarcinoma cells were plated (3×10^3 cells/well) into a 96-well plate overnight. After MTX incubation for 48 hours, choriocarcinoma cells were incubated with BrdU (10 μM, 2 hours). Incorporated BrdU was measured at OD_{450} using a Multiskan MK3 microplate reader.

Protein Extraction and Western Blotting
Western blotting was conducted as we described previously.[13] Briefly, Cells were washed in cold PBS and lysed in a radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Phenylmethanesulfonyl fluoride (PMSF), 1

mM Ethylenediamine tetra-acetic acid (EDTA), 5 mg/mL aprotinin, 5 mg/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The cell extract with 25 μg proteins was loaded on a 10% or 15% SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was incubated for 1 h in Tris-buffered saline (TBS) containing 5% skim milk. After blotting with primary antibody, the protein expression was revealed with the appropriate horseradish peroxidase–labeled secondary antibody, which was detected using ECL chemiluminescence. Mouse anti-human β-Actin was used as loading control.

MDC Incorporation Assay

MDC incorporation assay was conducted to measure the level of intracellular autophagy as described previously.[22] Choriocarcinoma cells were incubated with 50 μM MDC for 10 min. The incorporated MDC was detected (excitation 380 nm; emission 525nm) in F97Pro fluorospectrometer (Lengguang Technology, Shanghai, China).

Transmission Electron Microscopy

To demonstrate the autophagic morphology in choriocarcinoma cells, we performed transmission electron microscopy as described previously.[22] JEG3 and JEG3/MTX cells were fixed with ice-cold glutaraldehyde (2% in 0.1 M cacodylate buffer, pH 7.4) for 30 min. After fixation, the samples were postfixed in 1% OsO4 in the same buffer for 1 h and then subjected to the electron microscopic analysis. Representative areas were chosen for ultrathin sectioning and viewed with a Philips CM120 transmission electron microscope.

Immunofluorescence Staining

JEG3 and JEG3/MTX cells were seeded on the coverslips and grown overnight. Cells were fixed in 4% paraformaldehyde, permeated with 0.25% Triton X-100, blocked with 3% normal goat serum, stained with LC3 antibody overnight, and labeled with goat anti-rabbit IgG conjugated with tetramethylrhodamine (TRITC) (Zymed Laboratories, CA, USA). The cells were counterstained with sealant containing 40.6-diamidino-2-phenylindole (DAPI) (Meilunbio, Dalian, China) and examined under an Olympus BX40 fluorescence microscope (Olympus, Japan).

Statistical Analysis

SPSS 16.0 was used to conduct the statistical analysis. Data were expressed as mean ± standard deviation. Student's *t* test was conducted to compare the means of two groups. One-way ANOVA analysis was used to compare the means of three or more groups. $P < 0.05$ was considered statistically significant for all the statistical tests.

Results
SLAMF1 Expression is Increased in MTX-Resistant Cell Models

In our previous proteomics study,[13] we identified multiple genes/pathways potentially involved in MTX resistance in JEG3:JEG3/MTX cell models. SLAMF1 was shown to be the top protein highly enriched in JEG3/MTX subline (P<0.05; Figure 1A and B). Consistently, Western blotting analysis revealed an increase in SLAMF1 expression in MTX-resistant sublines compared to parental JEG3 and JAR cells, respectively (Figure 1C).

Knockdown of SLAMF1 Sensitized MTX-Resistant Choriocarcinoma Cells to MTX Treatment

To examine the effect of SLAMF1 on the drug response of JEG3/MTX and JAR/MTX cells to MTX, we depleted SLAMF1 expression through shRNA-mediated knockdown. The expression of SLAMF1 was mostly reduced by shRNA targeting SLAMF1 compared to scramble (Scr) control (Figure 2A). Knockdown of SLAMF1 enhanced MTX chemosensitivity, as IC_{50} value was lower in shSLAMF1 group than in Scr control in MTX-resistant JEG3 and JAR sublines (Figure 2B). In addition, the effect of SLAMF1 on cellular proliferative potential was examined by BrdU incorporation assay in JEG3/MTX and JAR/MTX cells. The shSLAMF1 group was found less BrdU incorporation compared with Scr group after incubation with MTX (10 μM) for 48 hours (Figure 2C). Consistently, knockdown of SLAMF1 reduced soft agar clonogenesis in JEG3/MTX and JAR/MTX cells after MTX treatment (10 μM), as compared with Scr control (Figure 2D).

Over-Expression of SLAMF1 Promotes Resistance to MTX in Choriocarcinoma Cells

We next overexpressed SLAMF1 in order to examine its function in JEG3 and JAR cells. SLAMF1
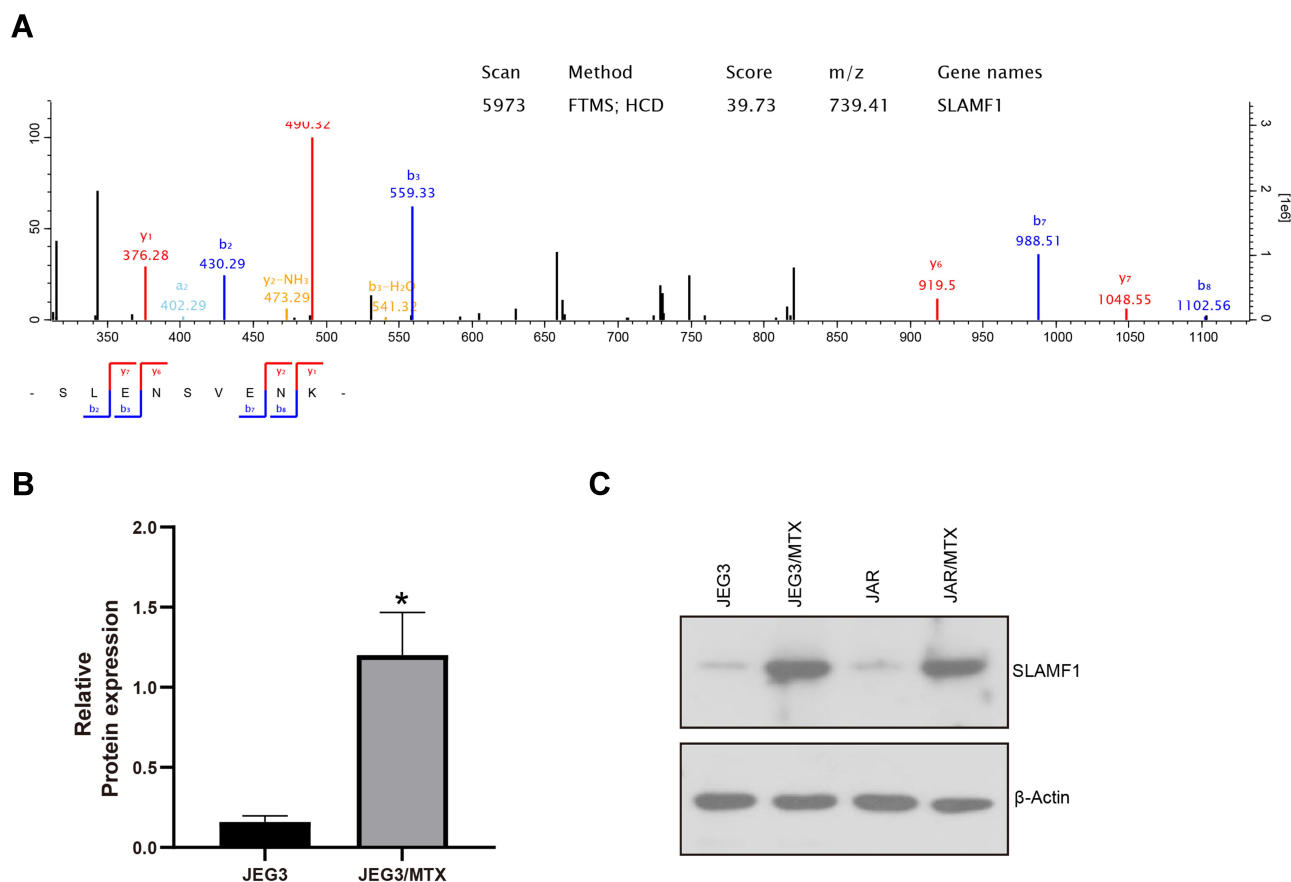
Figure 1 SLAMF1 expression is increased in MTX-resistant choriocarcinoma cells. (**A**). The mass spectrum of SLAMF1 unique peptide (SLENSVENK) identified by TMT labeling and LC-MS/MS. (**B**). Relative expression of SLAMF1 protein in JEG3 and its MTX-resistant subline JEG3/MTX. *P<0.05. (**C**). Western blotting validation on SLAMF1 expression in MTX-resistant choriocarcinoma sublines and their parental cell lines. β-actin was used as loading control.

expression was apparently increased in this SLAMF1 group compared to empty vector (EV) control (Figure 3A). Overexpression of SLAMF1 enhanced chemoresistance to MTX, as IC_{50} value was higher in SLAMF1 group than in EV control in JEG3 and JAR cells (Figure 3B). In addition, the effect of SLAMF1 on cellular proliferative potential was examined by BrdU incorporation assay in JEG3 and JAR cells. The SLAMF1 group was found more BrdU incorporation compared with EV group after incubation with MTX (3 μM) for 48 hours (Figure 3C). Consistently, overexpression of SLAMF1 increased soft agar clonogenesis in JEG3 and JAR cells after MTX treatment (3 μM), as compared with EV control (Figure 3D).

Autophagy Might Be Involved in MTX Resistance in Choriocarcinoma Cells

SLAMF1 is a crucial regulator of autophagy in chronic lymphocytic leukemia cells,[20] which might implicate autophagy as possible mechanism of chemoresistance to MTX in choriocarcinoma. As shown in Figure 4A, increased protein levels of autophagy-related genes LC3-II and p62 were observed in MTX-resistant sublines compared to parental choriocarcinoma cells, respectively. Consistently, More MDC incorporation was noted in MTX-resistant sublines than that in parental choriocarcinoma cells (Figure 4B). Electron microscopic observation on the ultrastructural features revealed enormous autophagic vacuoles in JEG3/MTX cells compared to JEG3 (Figure 4C). Consistently, punctate LC3 immunostaining was seen in JEG3/MTX, which represents typical autophagy morphology (Figure 4D). Cell viability in MTX-resistant sublines was greatly reduced in MTX plus 3-MA group or MTX plus bafilomycin, group, as compared with either drug alone (Figure 4E). These results suggested that autophagy might serve as a prosurvival mechanism in MTX-resistant choriocarcinoma cells.
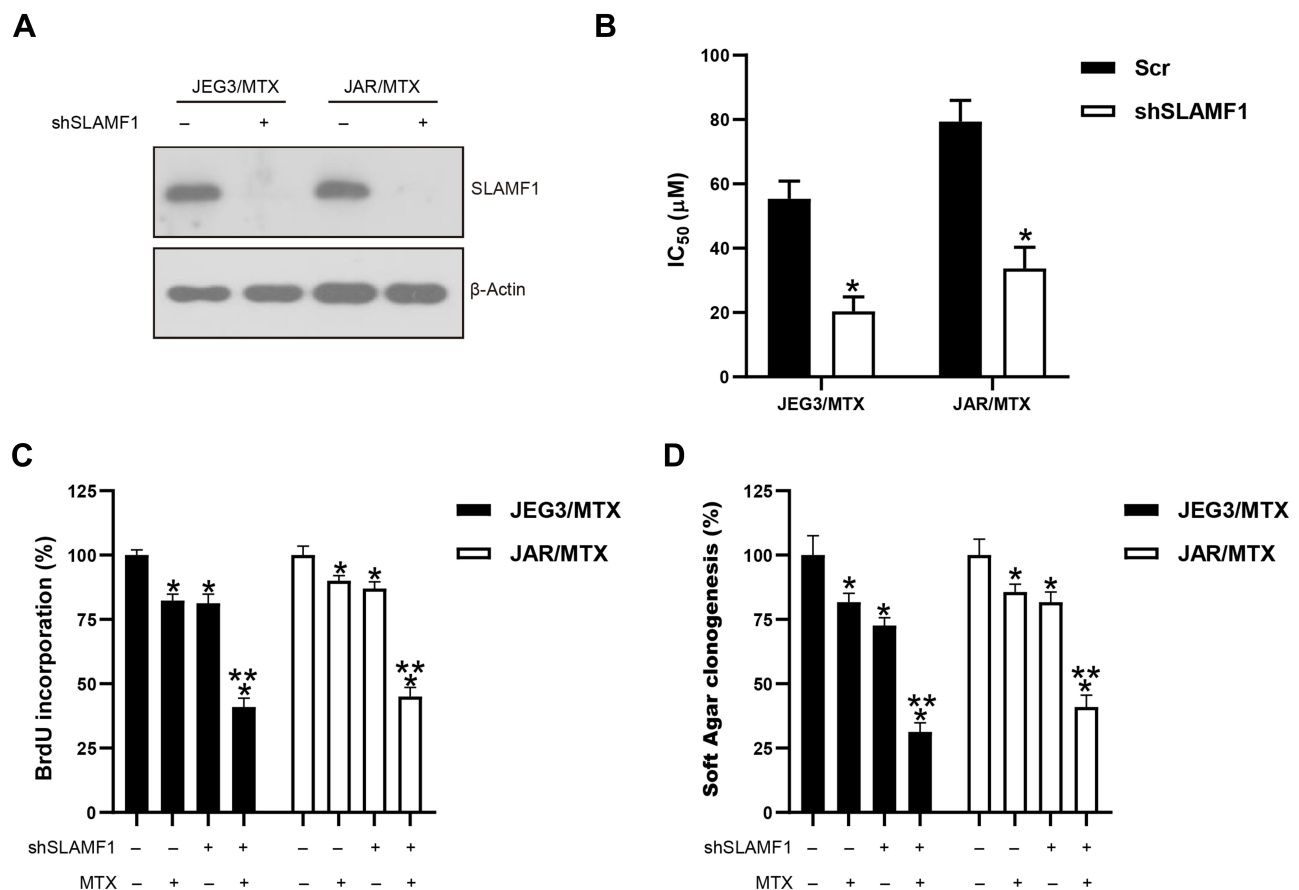
Figure 2 Knockdown of SLAMF1 enhanced MTX chemosensitivity in MTX-resistant choriocarcinoma cell lines. (**A**). SLAMF1 expression was markedly attenuated by gene-specific shRNA in JEG3/MTX and JAR/MTX cells. (**B**). Knockdown of SLAMF1 increased MTX sensitivity in JEG3/MTX and JAR/MTX cells. The cell viability in Scr or shSLAMF1 without MTX treatment was regarded as 100%, respectively. n=3,*P<0.01. (**C**). Knockdown of SLAMF1 attenuated the proliferative potential of JEG3/MTX and JAR/MTX cells after MTX treatment for 48 hours. The BrdU incorporation in Scr without MTX treatment was regarded as 100%. n=3, *P<0.05, as compared with Scr without MTX treatment. **P<0.05, as compared with Scr with MTX treatment. (**D**). Knockdown of SLAMF1 impaired soft agar clonogenesis after MTX treatment in JEG3/MTX and JAR/MTX cells. The colony formation in Scr group without MTX treatment was regarded as 100%, respectively. n=3, *P<0.05, as compared with Scr without MTX treatment. **P<0.05, as compared with Scr with MTX treatment.

SLAMF1 Regulates Autophagy in Choriocarcinoma Cell Lines

We next examined the effect of SLAMF1 on autophagy level in choriocarcinoma cells. Knockdown of SLAMF1 attenuated the expression of autophagy-related genes (LC3-II and p62) and inhibited MDC incorporation compared to Scr control in JEG3/MTX and JAR/MTX cells (Figure 5A and B). In contrast, over-expression of SLAMF1 induced the expression of autophagy-related genes (LC3-II and p62) and induced MDC incorporation compared to empty vector (EV) control in JAR and JEG3 cells (Figure 5C and D).

Depletion of SLAMF1 Induced Apoptosis in JEG3/MTX and JAR/MTX Cells After MTX Treatment

We depleted SLAMF1 expression in MTX-resistant choriocarcinoma cells in order to elucidate the role of

autophagy on cellular apoptosis. Western blotting showed that SLAMF1 depletion could considerably reduce LC3-II and p62 levels in JEG3/MTX (Figure 6). Meanwhile, the level of apoptosis-related cleaved caspase-3 was significantly increased after MTX treatment in shSLAMF1 group compared to Scr control (Figure 6).

Discussion

The acquisition of chemoresistance to methotrexate (MTX) still remains one of the major challenges for choriocarcinoma treatment. Exploring the mechanism of MTX resistance would help to develop therapeutic strategy to combat MTX-resistant choriocarcinoma. The signaling lymphocytic activation molecule (SLAM) family has been shown to regulate inflammation and immune response.[23] Recently, SLAM genes have been implicated in drug response regulation in cancerous cells. Fouquet
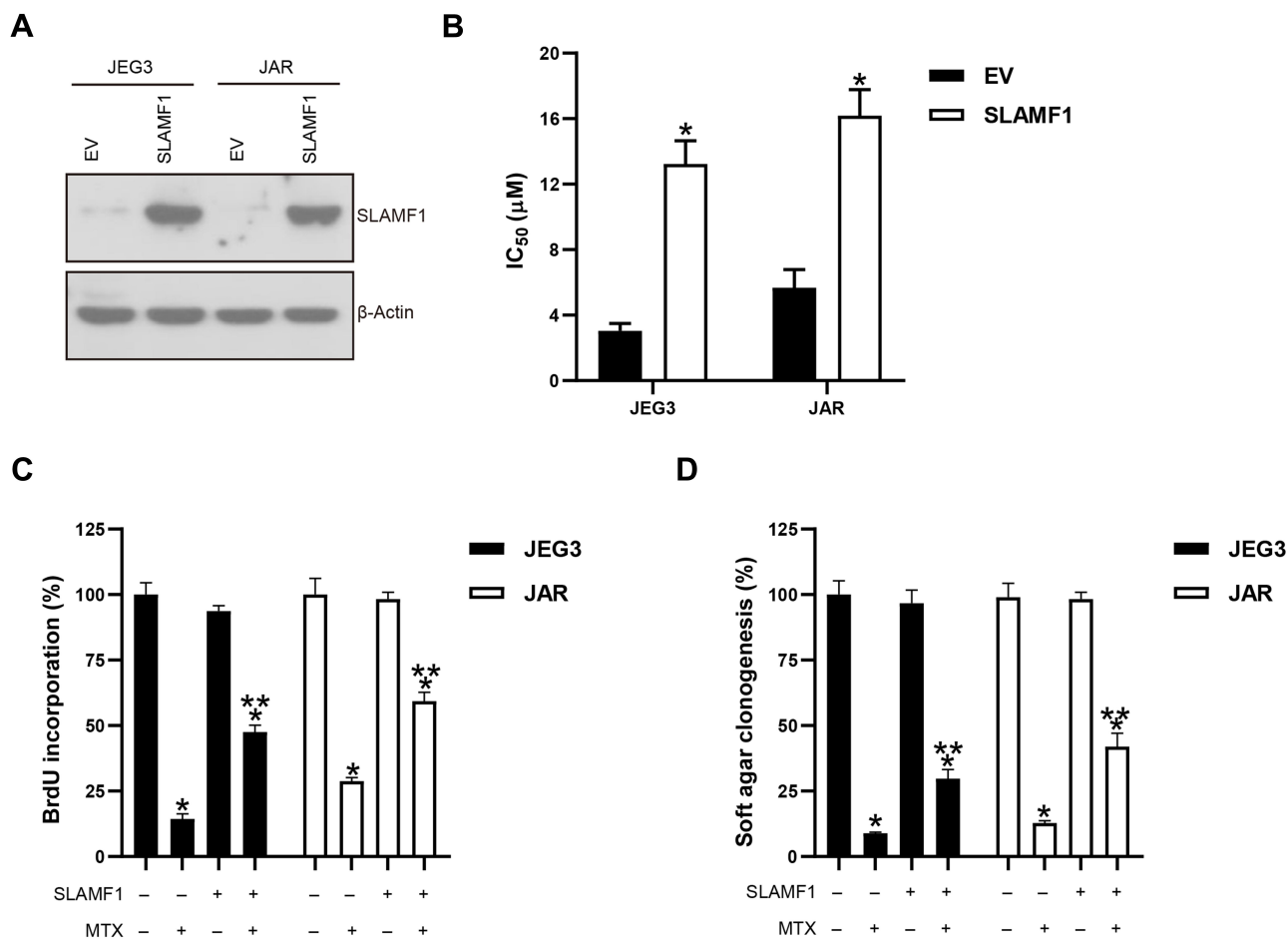
Figure 3 Over-expression of SLAMF1 promoted chemoresistance to MTX in JEG3 and JAR cells. (**A**). SLAMF1 expression was considerably increased in JEG3 and JAR cells. (**B**). Over-expression of SLAMF1 promoted chemoresistance to MTX in JEG3 and JAR cells. n=3, *P<0.05. (**C**). Over-expression of SLAMF1 enhanced the proliferative potential of choriocarcinoma cells after MTX treatment. The BrdU incorporation in EV of each cell line without MTX treatment was regarded as 100%, respectively. n=3, *P<0.05, as compared with EV without MTX treatment; **P<0.05, as compared with EV with MTX treatment. (**D**). Over-expression of SLAMF1 rescued soft agar clonogenesis after MTX treatment in choriocarcinoma cell lines. The colony formation in EV of each cell line without MTX treatment was regarded as 100%, respectively. n=3, *P<0.05, as compared with EV without MTX treatment; **P<0.05, as compared with EV with MTX treatment.

et al showed that SLAMF3 inhibits liver cancer growth and might predict resistance to sorafenib.[24] Bologna et al showed that loss of SLAMF1 was associated with cellular response to fludarabine and ABT-737 in chronic lymphocytic leukemia cells.[20] Recently, our proteomic profiling identified SLAMF1 as a potential regulator of MTX resistance in JEG3/JEG3/MTX cell models.[13] Knockdown of SLAMF1 markedly attenuated cell viability and soft agar clonogenesis after incubation with MTX in JEG3/MTX and JAR/MTX cells. In contrast, overexpression of SLAMF1 rescued cell survival soft agar clonogenesis in JEG3 and JAR cells treated with MTX. Therefore, our findings might connect the novel function of the SLAMF1 with the modulation of drug resistance in choriocarcinoma.

Autophagy is an important cellular mechanism that recycles cytoplasmic components into lysosome for bulk degradation.[25,26] Physiologically, autophagy pathway is dramatically induced in order to survive from nutrient starvation and stress.[27,28] Autophagy could also be induced by cancer therapy, which contributes to cancer cell survival and the eventual recurrence of the disease.[29,30] Autophagy machinery could be activated in several cell models in response to MTX treatment. Xu et al showed that autophagy induction contributes to the resistance to methotrexate treatment in rheumatoid arthritis fibroblast-like synovial cells.[31] Shen et al revealed a survival mechanism of the switch from ER stress-induced apoptosis to autophagy via ROS-mediated JNK/p62 signals in methotrexate-resistant choriocarcinoma
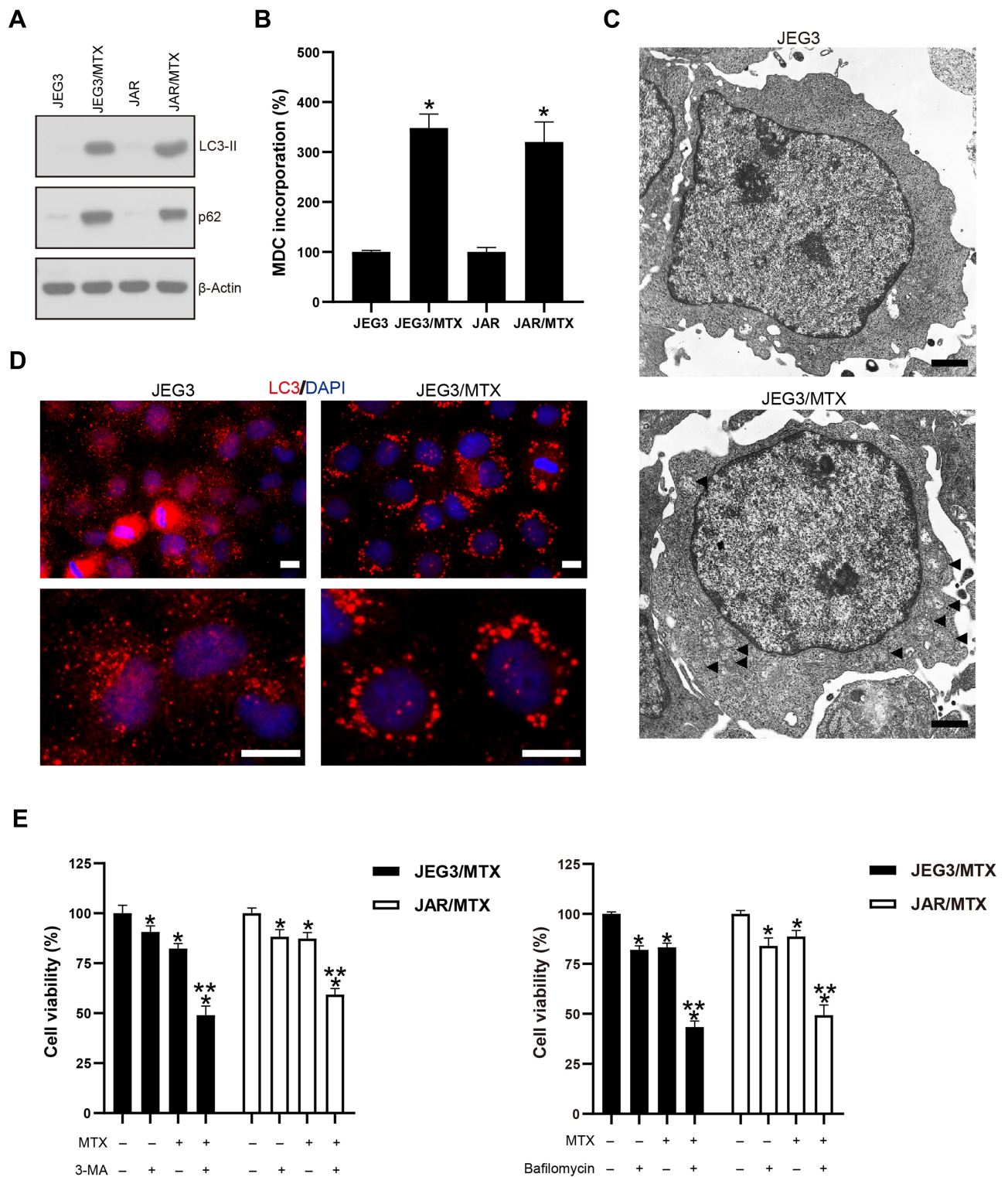
Figure 4 Autophagy might be involved in MTX resistance in MTX-resistant choriocarcinoma cells. (**A**). Western blotting analysis on the expression of autophagy-related genes LC3B and p62 in MTX-resistant choriocarcinoma sublines and parental cell lines. (**B**). MDC incorporation in MTX-resistant choriocarcinoma sublines and parental cell lines. n=3, *P<0.05. (**C**). Electron microscopic observation on the ultrastructural features of autophagy in JEG3 and JEG3/MTX cells. Black triangles indicated the autophagic vacuoles in JEG3/MTX cells. Bars: 1 μm. (**D**). Punctate LC3 immunostaining was seen in JEG3/MTX compared to JEG3, which represents typical autophagy morphology. Bars: 10 μm. (**E**). Autophagy inhibitor 3-MA and bafilomycin A1 enhanced MTX-induced cytotoxicity in MTX-resistant choriocarcinoma cells. n=3, *P<0.05, as compared with vehicle control. **P<0.05, as compared with either drug alone.
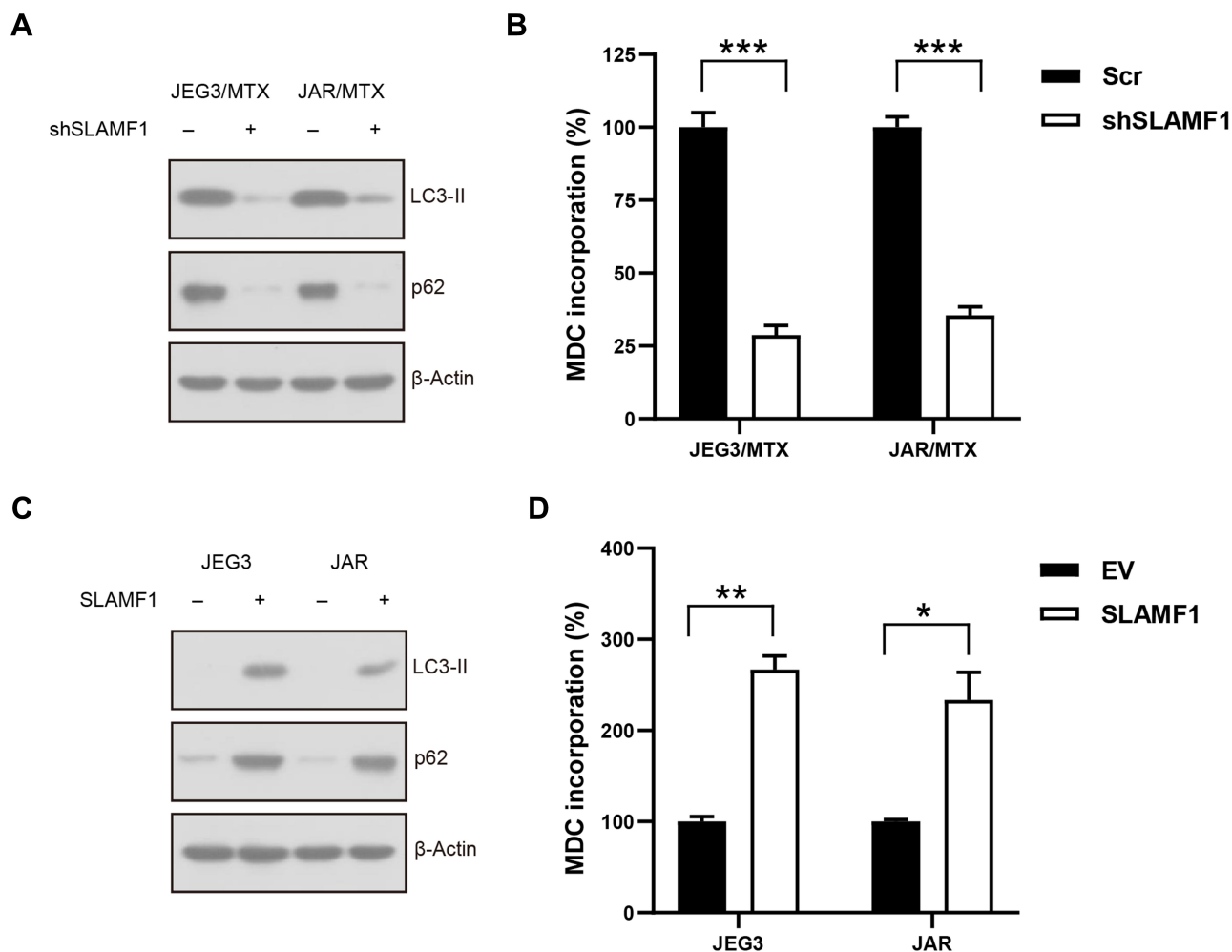
Figure 5 SLAMF1 regulates autophagy in choriocarcinoma cells. (**A**). Knockdown of SLAMF1 attenuated the expression of autophagy-related genes (LC3-II and p62) in MTX-resistant choriocarcinoma sublines. (**B**). MDC incorporation in MTX-resistant choriocarcinoma sublines with or without SLAMF1 depletion. n=3, ***P<0.001. (**C**). SLAMF1 over-expression increased the expression of autophagy-related genes (LC3-II and p62) in choriocarcinoma cells. β-actin was used as loading control. (**D**). MDC incorporation in choriocarcinoma cell lines with or without SLAMF1 expression. n=3, *P<0.05; **P<0.01.

cells.[32] Similarly, our findings showed that autophagy-related genes (LC3-II, p62) were apparently increased in MTX-resistant choriocarcinoma cells compared to parental cell lines. Further, autophagy inhibitor 3-MA and Bafilomycin could enhance MTX-induced cytotoxicity in MTX-resistant choriocarcinoma cells. These results suggested that autophagy-related mechanisms might play an important role in MTX resistance in choriocarcinoma cells.

Recently, SLAMF1 has been shown to be a critical regulator of autophagy induced by fludarabine or ABT-737 in chronic lymphocytic leukemia cells, which might connect SLAMF1-regulated autophagy with drug response in cancer cells.[20] However, the role of SLAMF1 in regulating MTX resistance in choriocarcinoma cells still remains unknown. Herein, we showed that manipulation of SLAMF1

expression could modulate autophagy in choriocarcinoma cell models, suggesting SLAMF1 might be a critical regulator of autophagy in choriocarcinoma cells. Further, autophagy regulated by SLAMF1 might be an important prosurvival mechanism, as knockdown of SLAMF1 suppressed autophagy and induced apoptosis in JEG3/MTX and JAR/MTX cells treated with MTX. Ma et al showed that SLAMF1 could recruit a Beclin-1/Vps34/UVRAG complex under oxidative stress condition, which leads to activation of the membrane NADPH oxidase 2 complexes.[33] Therefore, SLAMF1 might regulate autophagy-related pathway to activate antioxidant enzymes, which would be crucial in mediating chemoresistance to MTX in choriocarcinoma cells.

In conclusion, our findings suggest that SLAMF1 might promote MTX resistance via activating protective
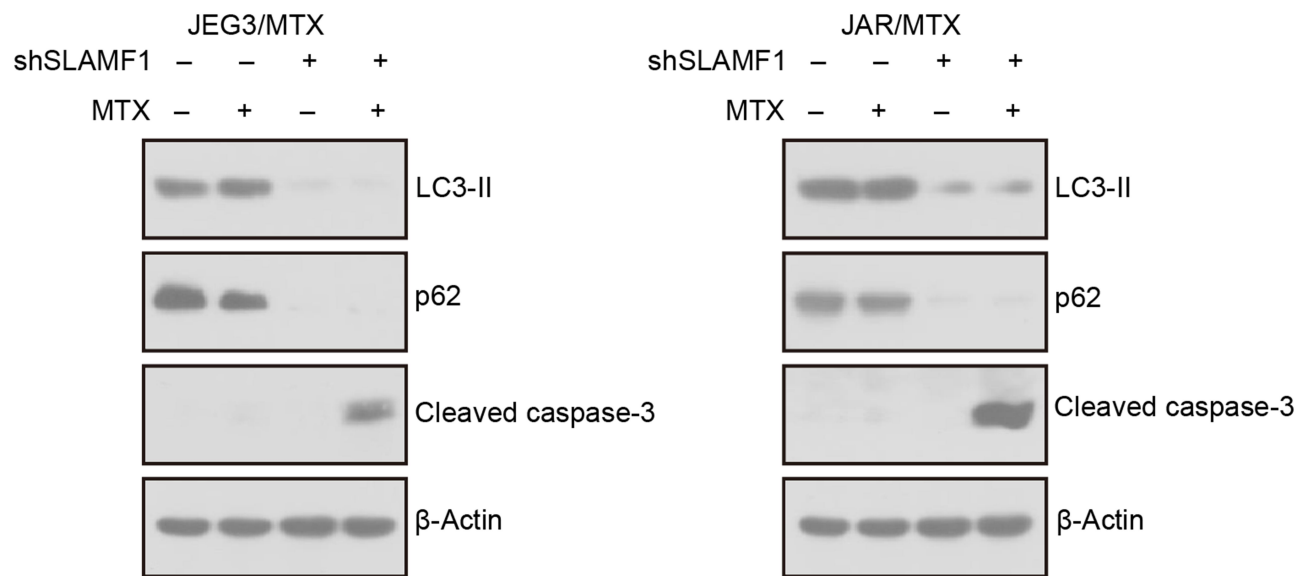
Figure 6 Depletion of SLAMF1 induced apoptosis in MTX-resistant choriocarcinoma cells treated with MTX. Western blotting analysis detects the expression of LC3-II, p62 and cleaved caspase-3 after MTX treatment in shSLAMF1 or Scr group.

autophagy in choriocarcinoma cell lines. Targeting SLAMF1 might be a useful therapeutic strategy to sensitize choriocarcinoma cells to MTX-based regimens.

Acknowledgments

Disclosure

The authors have no competing interests to declare.

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