European Review for Medical and Pharmacological Sciences 2018; 22: 6008-6014

Jagged2 promotes cancer stem cell properties of triple negative breast cancer cells and paclitaxel resistance via regulating microRNA-200

C.-Y. LI[1], K.-L. MIAO[1], Y. CHEN[2], L.-Y. LIU[1], G.-B. ZHAO[1], M.-H. LIN[3], C. JIANG[1]

[1]Department of Oncological Surgery, Fujian Medical University Affiliated MinDong Hospital, Fuan, China
[2]Department of Medical Oncology, Fujian Medical University Affiliated MinDong Hospital, Fuan, China
[3]Department of Pathology, Fujian Medical University Affiliated MinDong Hospital, Fuan, China

Abstract. – OBJECTIVE: To investigate the role of Jagged2 in triple negative breast cancer (TNBC) and its underlying mechanism.

PATIENTS AND METHODS: Breast cancer tissues of patients diagnosed with TNBC in Fujian Medical University Affiliated MinDong Hospital from January 2015 to September 2017 were selected. TNBC patients were divided into the paclitaxel-resistant group (n=34) and non-resistance group (n=11). Jagged2 expression in paclitaxel-resistant group and non-resistance group before and after treatment was detected by quantitative Real-time-polymerase chain reaction (qRT-PCR), respectively. After Jagged2 knockdown in paclitaxel-resistant MDA-MB-231 cells (MDA-MB-231/TXR), expression of CD44+CD24-ESA+ subset was detected by flow cytometry. MicroRNA-200 expression was detected after Jagged2 knockdown in MDA-MB-231/TXR cells.

RESULTS: Jagged2 was highly expressed in paclitaxel-resistant TNBC tissues and cells. Jagged2 expression was found to be associated with cancer stem cell (CSC) properties of TNBC cells. Knockdown of Jagged2 inhibited CSC properties and paclitaxel resistance, whereas upregulated microRNA-200 expression. The inhibited CSC properties and paclitaxel resistance induced by Jagged2 knockdown were reversed by microRNA-200 knockdown.

CONCLUSIONS: Jagged2 maintains CSC properties of TNBC cells and paclitaxel resistance via regulating microRNA-200.

Key Words:
Triple negative breast cancer, Jagged2, Drug resistance, Cancer stem cell properties.

Introduction

Breast cancer is one of the most common malignancies in women[1]. With the in-depth studies, breast cancer is considered as a systemic disease. Systemic combination treatment has become the preferred treatment for breast cancer instead of surgical resection. Since the molecular classification of breast cancer has been proposed, corresponding treatment based on the specific molecule effectively prolongs the overall survival of breast cancer patients[2,3]. Triple negative breast cancer (TNBC) is a special type of breast cancer in which expressions of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) are all negative[4]. Due to the lack of endocrine therapy and anti-HER2 targeted drug therapy, the currently treatment for TNBC is still based on chemotherapy. Hence, it is of great importance of chemotherapy in the comprehensive treatment for TNBC. At present, the first-line treatments for TNBC chemotherapy are mainly anthracycline and taxane drugs[5-7]. Paclitaxel belongs to a kind of taxane antimicrotubule drug. Paclitaxel has been widely applied in breast cancer chemotherapy because of its unique role in promoting microtubule polymerization and stabilizing polymerized microtubules. Drug resistance in tumor chemotherapy is common, leading to local recurrence or distant metastasis, which seriously affects the prognosis of tumor patients[8-10]. Since breast cancer patients with ER-positive or HER2-positive could receive endocrine therapy or targeted drug therapy, the

Corresponding Author: Chuan Jiang, BM; e-mail: jc6336946@163.com

effect of chemotherapy resistance on the prognosis of breast cancer patients is relatively small. However, current treatment for TNBC patients is still not effective enough, it is urgent to explore the mechanism of drug resistance to improve clinical outcomes of TNBC patients[11]. Previous studies have demonstrated that the Notch ligands Jagged1 and Jagged2 are associated with poor prognosis of TNBC[12]. Jagged2 is closely related to CSC properties and drug resistance in ovarian cancer cell lines[13]. In this work, TNBC cell line MDA-MB-231 was selected to establish a paclitaxel-resistant cell line using intermittent stimulation. We aim to explore the role of Jagged2 in paclitaxel resistance and CSC properties of TNBC cells.

Patients and Methods

Sample Collection

Breast cancer tissues of patients diagnosed with TNBC in Fujian Medical University Affiliated MinDong Hospital from January 2015 to September 2017 were selected. TNBC patients were divided into the paclitaxel-resistant group (n=34) and non-resistance group (n=11), respectively based on their one-year follow-up data. All patients signed informed consent before the surgery. This study was approved by the Ethics Committee of Fujian Medical University Affiliated MinDong Hospital.

Cell Culture and Transfection

TNBC cell line (MDA-MB-231) was cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum) (Gibco, Grand Island, NY, USA) and incubated in a 5% CO_2 incubator at 37°C. MDA-MB-231 cells in logarithmic growth phase were seeded in the 6-well plates. When the cell confluence was up to 60%, cell transfection was performed following the manufacturer's instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Tokyo, Japan). The relative gene expression was calculated using $2^{-\Delta Ct}$ method. Primers used in the study were as follows: Jagged2, forward: 5′-CCGGATAACCTGGAGGGTGACTATTCTCGAGAATAGTCACCCTCCAGGTTATTTTTTG-3′, reverse: 5′-AATTCAAAAAATAACCTGGAGGGTGACTATTCTCGAGAATAGTCACCCTCCAGGTTAT-3′; MicroRNA-200, forward: 5′-AATAATGGATCCCAGGACACTTCGGCCC-3′, reverse: 5′-ATGTGTGAATTCAAAAACAGGAGGCCTTG-3′; GAPDH, forward: 5′-AGAAGGCTGGGGCTCATTTG-3′, reverse 5′-AGGGGCCATCCACAGTCTTC-3′.

Western Blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) lysis buffer containing protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) to harvest total cellular protein. The protein concentration of each cell lysate was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein sample was loaded onto a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after being separated. After blocked with skim milk, membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 2-3 h at room temperature. Finally, an image of the protein band was captured by the Tanon detection system using ECL (electrochemiluminescence) reagent (Thermo-Fisher, Waltham, MA, USA).

Flow Cytometry

Cell supernatant was collected and preserved in labeled flow tube. Cells were then digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin and washed with PBS (phosphate-buffered saline) twice. 200 μL of binding buffer containing calcium ion and corresponding antibodies were added for incubation in dark, followed by flow cytometry detection of cell subset expression.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk,

NY, USA) was used for data analysis and GraphPad Prism 6.0 (La Jolla, CA, USA) was introduced for figure editing. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared using the *t*-test. $p < 0.05$ considered the difference was statistically significant (*$p < 0.05$, **$p < 0 .01$, ***$p < 0.001$).

Results

Jagged2 was Highly Expressed in TNBC

Enrolled TNBC patients were divided into the paclitaxel-resistant group (n = 34) and non-resistance group (n = 11). QRT-PCR results showed that Jagged2 was highly expressed in paclitaxel-resistant group than that of non-resistance group (Figure 1A). Moreover, Jagged2 expression was upregulated in paclitaxel-resistant group after treatment, whereas no significant difference was found in non-resistance group before and after treatment (Figure 1B). Subsequently, we screened paclitaxel-resistant cell line by paclitaxel induction in MDA-MB-231 cells. CCK-8 results demonstrated that the tolerance rate to different doses of paclitaxel was higher in MDA-MB-231/TXR cells than that of parental MDA-MB-231 cells (Figure 1C). Jagged2 expression was also higher in MDA-MB-231/TXR cells compared with that of parental cells (Figure 1D).

Jagged2 Was Highly Expressed in TNBC Cells With CSC Properties

Previous studies showed that Jagged2 was overexpressed in breast cancer cells with CSC properties. In our study, we isolated $CD44^{+}CD24^{-}$

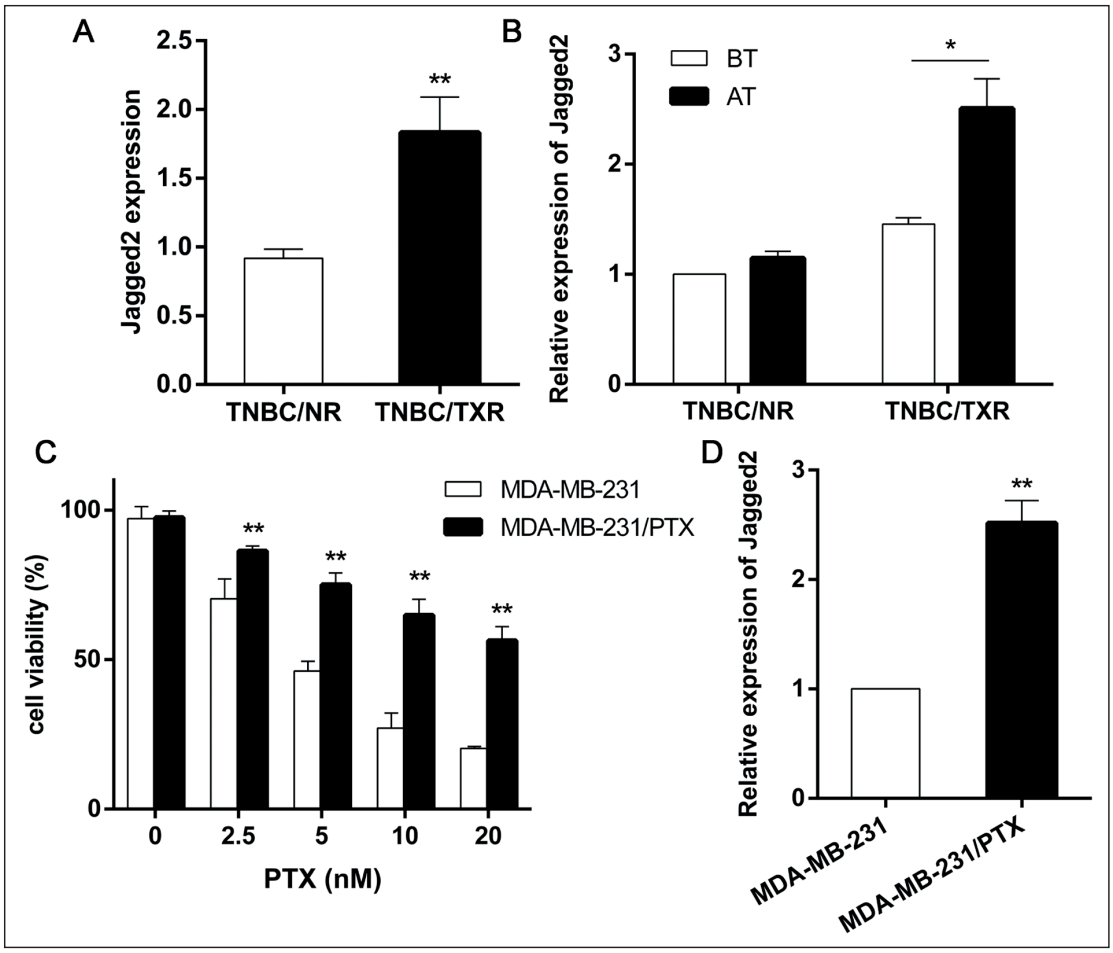

Figure 1. Jagged2 was highly expressed in TNBC. ***A,*** Jagged2 was highly expressed in paclitaxel-resistant group than that of non-resistance group. ***B,*** Jagged2 expression was upregulated in paclitaxel-resistant group after treatment, whereas no significant difference was found in non-resistance group. ***C,*** CCK-8 results demonstrated that the tolerance rate to different doses of paclitaxel was higher in MDA-MB-231/TXR cells than that of parental MDA-MB-231 cells. ***D,*** Jagged2 expression was higher in MDA-MB-231/TXR cells compared with that of parental cells.

ESA^+ cells in TNBC tissues by flow cytometry. The data indicated that expression level of $CD44^+CD24\text{-}ESA^+$ subset was remarkably higher in TNBC compared with that of other breast cancer (Figure 2A). We also detected Jagged2 expression in epithelioid cells and mesenchymal cells. It was found that Jagged2 was highly expressed in mesenchymal cells than that of epithelioid cells (Figure 2B). Besides, MDA-MB-231 cells here were cultured as adherent cells and microspheres, respectively. Upregulated Jagged2 was found in MDA-MB-231 cells cultured as microspheres, which was remarkably decreased by collagenase-1 induction (Figure 2C). Moreover, CSC-related genes were also highly expressed in microsphere MDA-MB-231 cells than those of adherent cells (Figure 2D).

Jagged2 Knockdown Inhibited CSC Properties and Paclitaxel Resistance of TNBC

We first constructed si-Jagged2 and verified its transfection efficacy in TNBC cells (Figure 3A). After Jagged2 knockdown, the subset of $CD44^+CD24\text{-}ESA^+$ cells was analyzed by flow cytometry. Relative results indicated that the proportion of $CD44^+CD24\text{-}ESA^+$ cell subset was remarkably decreased (Figure 3B). We also detect-

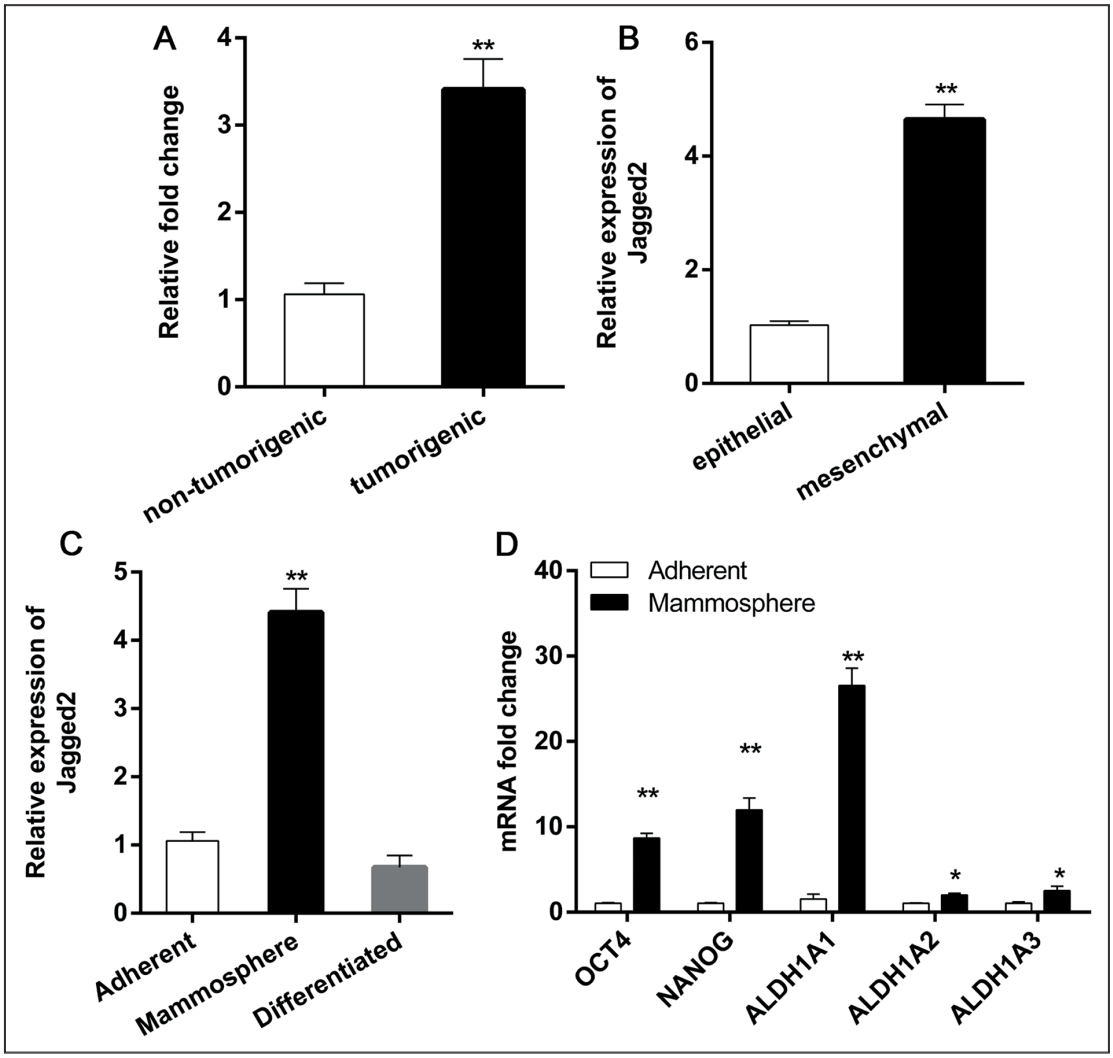

Figure 2. Jagged2 was highly expressed in TNBC cells with CSC properties. ***A,*** Expression level of $CD44^+CD24\text{-}ESA^+$ cells was remarkably higher in TNBC compared with that of other types of breast cancer. ***B,*** Jagged2 was highly expressed in mesenchymal cells than that of epithelioid cells. ***C,*** Upregulated Jagged2 was found in MDA-MB-231 cells cultured as microspheres, which was remarkably decreased by collagenase-1 induction. ***D,*** CSC-related genes were highly expressed in microsphere MDA-MB-231 cells.

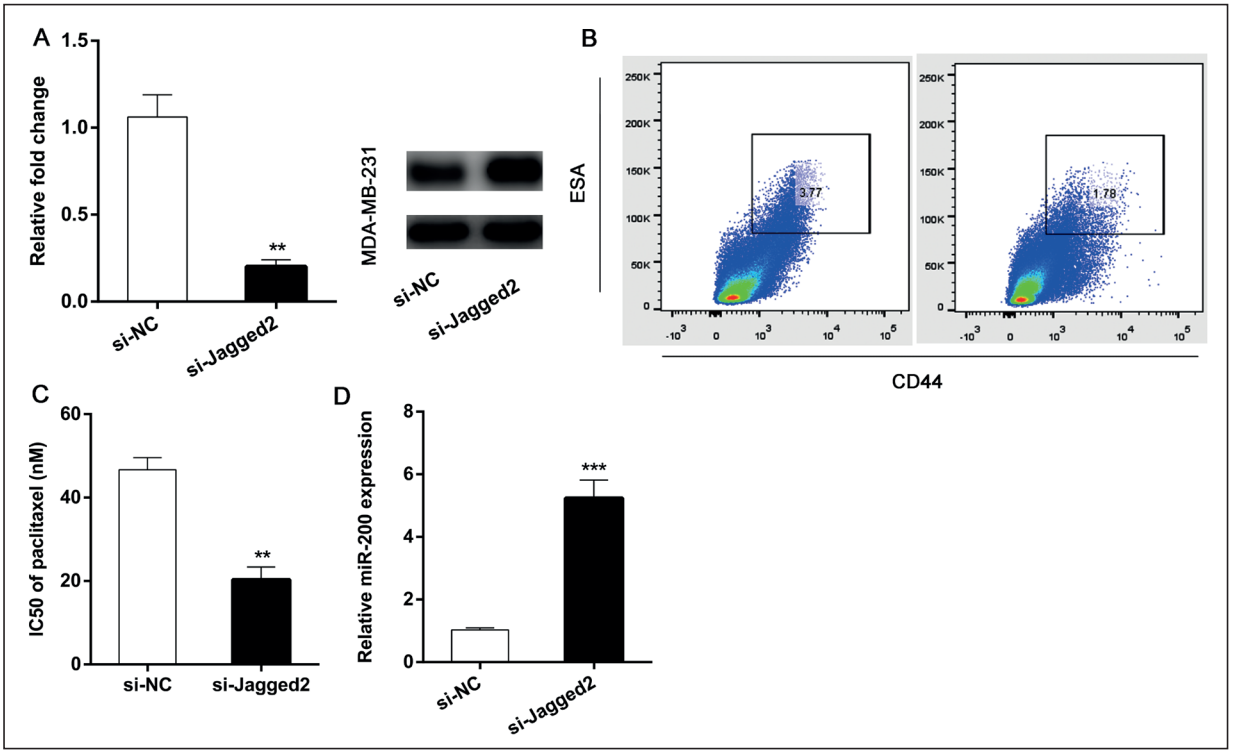

Figure 3. Jagged2 knockdown inhibited CSC properties and paclitaxel resistance of TNBC. ***A,*** Transfection efficacy of si-Jagged2. ***B,*** The proportion of $CD44^+CD24\text{-}ESA^+$ cell subset was remarkably decreased. ***C,*** IC_{50} of MDA-MB-231/TXR was remarkably reduced after knockdown of Jagged2. ***D,*** MicroRNA-200 expression was upregulated by transfection of si-Jagged2.

ed drug resistance of TNBC cells after Jagged2 knockdown. CCK-8 assay indicated that IC_{50} of MDA-MB-231/TXR was remarkably reduced after knockdown of Jagged2 (Figure 3C). Subsequently, microRNA-200 expression was upregulated by transfection of si-Jagged2 (Figure 3D).

Jagged2 Promoted CSC Properties and Paclitaxel Resistance of TNBC Via Upregulating microRNA-200

The above results showed that knockdown of Jagged2 upregulated microRNA-200 expression. To further determine the regulatory effect of Jagged2 on microRNA-200, MDA-MB-231/TXR cells were co-transfected with si-Jagged2 and anti-microRNA-200 (Figure 4A). The proportion of $CD44^+CD24\text{-}ESA^+$ cell subset and IC_{50} of MDA-MB-231/TXR were reversed by anti-microRNA-200 transfection (Figure 4B and 4C).

Discussion

Breast cancer is a systemic disease and chemotherapy exerts an important role in the treatment of breast cancer. In current treatment, multidrug resistance (MDR) is the greatest difficulty in tumor treatment[11, 14]. With the rapid development of molecular biology technology, gene therapy has gradually become an essential part of the tumor treatment. Therefore, drugs to reverse MDR based on the specific target have been well recognized. In particular, TNBC treatment is lacked of effective molecular target[5, 15]. In recent years, some potential targets for TNBC have been found, including EGFR (epidermal growth factor receptor), c-MET (hepatocyte growth factor receptor), VEGFR (vascular endothelial growth factor receptor) and FGFR (fibroblast growth factor receptor)[15-17]. Although researches on the mechanism of drug resistance of breast cancer have been widely conducted, other potential mechanisms still need to be explored to develop new therapeutic methods. Drug resistance protein, as a drug pump, expels cytotoxic drug by ATP-dependent hydrolysis, thereafter reducing the drug accumulation in cells[18-20]. However, the specific molecular mechanism of drug resistance of TNBC still remains unclear, which requires for further investigations. It is reported

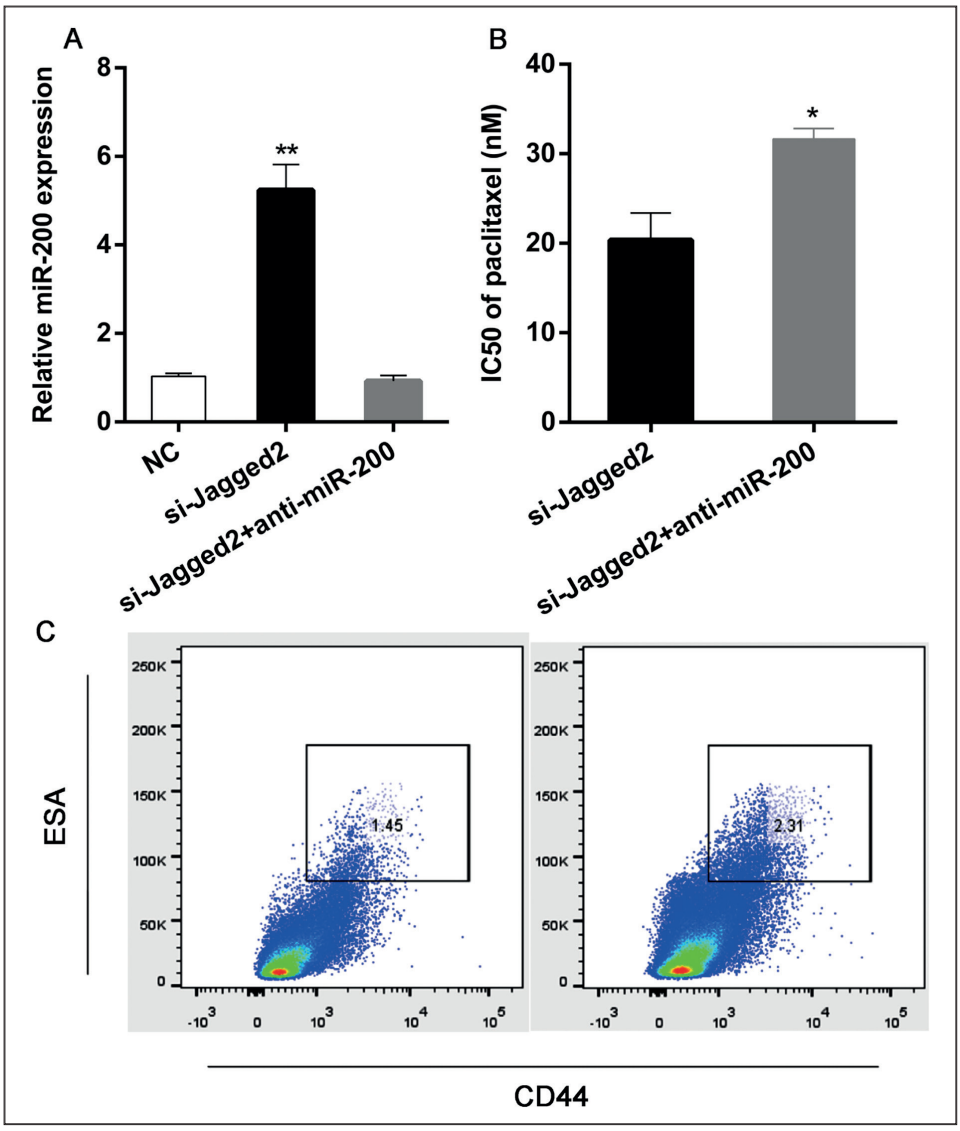

Figure 4. Jagged2 promoted CSC properties and paclitaxel resistance of TNBC *via* upregulating microRNA-200. ***A,*** MDA-MB-231/TXR cells were co-transfected with si-Jagged2 and anti-microRNA-200. ***B, C,*** The proportion of $CD44^+CD24\text{-}ESA^+$ cell subset (***B***) and IC_{50} (***C***) of MDA-MB-231/TXR were reversed by microRNA-200 knockdown.

that the Notch ligands Jagged1 and Jagged2 are associated with poor prognosis of TNBC[12]. In addition, Jagged2 is closely related to CSC and drug resistance in ovarian cancer cell lines. Our study found that Jagged2 expression was up-regulated in paclitaxel-resistant TNBC tissues and cells with CSC properties. Knockdown of Jagged2 inhibited CSC properties and paclitaxel resistance of TNBC, and upregulated microRNA-200 expression. The inhibited CSC properties and paclitaxel resistance in MDA-MB-231/TXR cells were reversed by microRNA-200 knockdown.

Conclusions

We showed that Jagged2 was highly expressed in TNBC, which maintains CSC properties of TNBC cells and paclitaxel resistance *via* regulating microRNA-200.

Funding Acknowledgements

This research was supported by grants from the Natural Science Foundation of Fujian Province, China (No. 2015J01575).

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
2) Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lonning PE, Brown PO, Borresen-Dale AL, Botstein D. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003; 100: 8418-8423.
3) Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. Nature 2000; 406: 747-752.
4) Bertucci F, Finetti P, Cervera N, Esterni B, Hermitte F, Viens P, Birnbaum D. How basal are triple-negative breast cancers? Int J Cancer 2008; 123: 236-240.
5) Kalimutho M, Parsons K, Mittal D, Lopez JA, Srihari S, Khanna KK. Targeted therapies for triple-negative breast cancer: combating a stubborn disease. Trends Pharmacol Sci 2015; 36: 822-846.
6) Andre F, Zielinski CC. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. Ann Oncol 2012; 23 Suppl 6: i46-i51.
7) Abramson VG, Lehmann BD, Ballinger TJ, Pietenpol JA. Subtyping of triple-negative breast cancer: implications for therapy. Cancer 2015; 121: 8-16.
8) Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006; 5: 219-234.
9) Marotta G, Muto T, Benincasa G, De Monaco A. Formyl peptide receptor 2 mediated chemotherapeutics drug resistance in colon cancer cells. Point of view from pharmacogenetics field. Eur Rev Med Pharmacol Sci 2018; 22: 1178-1179.
10) Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741-4751.
11) Choi YH, Yu AM. ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des 2014; 20: 793-807.
12) Zhu H, Bhaijee F, Ishaq N, Pepper DJ, Backus K, Brown AS, Zhou X, Miele L. Correlation of Notch1, pAKT and nuclear NF-kappaB expression in triple negative breast cancer. Am J Cancer Res 2013; 3: 230-239.
13) Kim MJ, Kim AR, Jeong JY, Kim KI, Kim TH, Lee C, Chung K, Ko YH, An HJ. Correlation of ALDH1 and Notch3 expression: clinical implication in ovarian carcinomas. J Cancer 2017; 8: 3331-3342.
14) Wu Q, Yang Z, Nie Y, Shi Y, Fan D. Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches. Cancer Lett 2014; 347: 159-166.
15) Bayraktar S, Gluck S. Molecularly targeted therapies for metastatic triple-negative breast cancer. Breast Cancer Res Treat 2013; 138: 21-35.
16) Ciriello G, Gatza ML, Beck AH, Wilkerson MD, Rhie SK, Pastore A, Zhang H, McLellan M, Yau C, Kandoth C, Bowlby R, Shen H, Hayat S, Fieldhouse R, Lester SC, Tse GM, Factor RE, Collins LC, Allison KH, Chen YY, Jensen K, Johnson NB, Oesterreich S, Mills GB, Cherniack AD, Robertson G, Benz C, Sander C, Laird PW, Hoadley KA, King TA, Perou CM. Comprehensive molecular portraits of invasive lobular breast cancer. Cell 2015; 163: 506-519.
17) Shah SP, Roth A, Goya R, Oloumi A, Ha G, Zhao Y, Turashvili G, Ding J, Tse K, Haffari G, Bashashati A, Prentice LM, Khattra J, Burleigh A, Yap D, Bernard V, McPherson A, Shumansky K, Crisan A, Giuliany R, Heravi-Moussavi A, Rosner J, Lai D, Birol I, Varhol R, Tam A, Dhalla N, Zeng T, Ma K, Chan SK, Griffith M, Moradian A, Cheng SW, Morin GB, Watson P, Gelmon K, Chia S, Chin SF, Curtis C, Rueda OM, Pharoah PD, Damaraju S, Mackey J, Hoon K, Harkins T, Tadigotla V, Sigaroudinia M, Gascard P, Tlsty T, Costello JF, Meyer IM, Eaves CJ, Wasserman WW, Jones S, Huntsman D, Hirst M, Caldas C, Marra MA, Aparicio S. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 2012; 486: 395-399.
18) Horsey AJ, Cox MH, Sarwat S, Kerr ID. The multidrug transporter ABCG2: still more questions than answers. Biochem Soc Trans 2016; 44: 824-830.
19) Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. AAPS J 2015; 17: 65-82.
20) Westover D, Li F. New trends for overcoming ABCG2/BCRP-mediated resistance to cancer therapies. J Exp Clin Cancer Res 2015; 34: 159.