

Autophagy regulates chemoresistance of gastric cancer stem cells via the Notch signaling pathway

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Abstract. – OBJECTIVE: Gastric cancer is the most common gastrointestinal malignancy and the leading cause of cancer-related deaths in East Asia. Increasing evidence has revealed that autophagy is closely associated with tumor initiation and progression. The present work aimed to investigate the role of autophagy in adjuvant chemotherapy for gastric cancer.

MATERIALS AND METHODS: Gastric cancer stem cells (CSCs) were isolated from gastric cancer cell lines using the cell surface markers CD44 and CD54 and cultured in a three-dimensional cell culture system. Western blotting was used to detect their protein expression levels in gastric CSCs. In addition, the cells were treated with inhibitors to investigate the underlying mechanisms of autophagy.

RESULTS: After isolation of gastric CSCs expressing CD44 and CD54, Western blot analysis showed that the levels of the autophagic marker LC3II were markedly enhanced in CD44+CD54+ gastric CSCs. Moreover, the ratio of LC3II/LC3I protein levels was higher in CD44+CD54+ gastric CSCs than in non-CSCs. By contrast, both a chemotherapeutic agent (5-fluorouracil) and autophagy inhibitor (chloroquine) exhibited an inhibitory effect on the cell viability of gastric CSCs, and their combination further enhanced such inhibitory effects. Mechanistically, the addition of Notch inhibitor decreased the cell viability of gastric CSCs treated with 5-fluorouracil and chloroquine. In addition, 5-fluorouracil and chloroquine both increased the expression of Notch1 in gastric CSCs.

CONCLUSIONS: These findings show that autophagy regulated drug sensitivity of gastric cancer cells through the Notch signaling pathway.

Key Words:

Gastric cancer, Stem cells, Autophagy, Notch signaling, Chemoresistance.

cancer-related deaths worldwide¹. Moreover, gastric cancer often reaches an advanced stage and is metastasized by the time symptoms appear. Thus, early detection of this disease is critical for limiting the local invasion and distant metastasis of gastric cancer cells². In addition, owing to multiple drug resistance, satisfying outcomes in patients with high grade of malignant gastric cancer are hardly achievable. Besides, the underlying mechanisms associated with chemoresistance in gastric cancer are not fully elucidated³.

Although cancer cells are heterogeneous in both morphology and function, a small set of tumor cells, termed cancer stem cells (CSCs), have the capacity to self-renew and initiate tumor growth⁴. CSCs have been observed in many solid tumors, such as breast cancer, brain cancer, colon cancer, and prostate cancer⁵⁻⁷. In gastric cancer, studies on CSCs are relatively recent. In 2009, Takaishi et al⁸ have screened a series of potential stem cell markers from a panel of human gastric cancer cell lines and demonstrated that CD44 may serve as a marker for gastric CSCs. In addition, Chen et al⁹ identified and elaborated on CSCs in tumor tissues and peripheral blood from patients with gastric adenocarcinoma by using the cell surface markers CD44 and CD54 (also known as ICAM-1).

Autophagy is a degradation process that involves the sequestration of cytosolic material, including organelles, into double membrane vesicles for delivery to the lysosome^{10,11}. Thus, autophagy prevents tissue damage and allows cells to sustain homeostasis under stressful conditions. Alterations in autophagy have been associated with diverse diseases, including cancer¹². Amassing evidence¹³ suggests that autophagy can suppress or promote tumors depending on the stage of the disease. A close relationship between autophagy and CSCs has been observed in several human neoplasms, such as colon cancer, breast cancer, and gastric cancer¹⁴⁻¹⁶.

In the present study, we first isolated CSCs in gastric cancer cells using CD44 and CD54 surface

Introduction

Gastric cancer is the fourth most common malignant tumor and the second highest cause of

markers. Subsequently, we evaluated the role of autophagy in the chemoresistance of gastric CSCs and further explored the underlying mechanism.

Materials and Methods

Cell Culture

The gastric cancer cell lines MGC-803 and MKN-45 were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and maintained at our institute. Cells were incubated at 37°C in a humidified environment containing 5% CO₂. All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin and 50 µg/mL streptomycin.

Cytofluorometric Cell Separation

For isolation of CSCs, cells were labeled with CD44 and CD54 antibodies (BD Biosciences, Franklin Lakes, NJ, USA) and sorted on a FACS Aria (BD Biosciences). After cytofluorometric sorting, cell purity was evaluated by flow cytometry using CD44/CD54 antibodies (BD Biosciences).

Spheroid Colony Formation Assay

The sorted CD44⁺CD54⁺ cells were inoculated into 48-well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Cells were treated with the chemotherapeutic agent 5-fluorouracil (5-FU, 1.25 µM) or the autophagy inhibitor chloroquine (CQ, 80 µM) alone or in combination at different time points. Subsequently, each well was examined under a light microscope (Olympus, Tokyo, Japan), and the spheroid colonies were counted.

Western Blot Analysis

Cells were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of each samples were loaded into 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of proteins onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, membranes were incubated with primary antibodies at 4°C overnight. After washing, secondary

antibodies used were goat anti-rabbit, conjugated to horseradish peroxidase (HRP) (Thermo Fisher Scientific, Waltham, MA, USA). Immunostained bands were detected by enhanced chemiluminescence (ECL: Pierce, Rockford, IL, USA).

Statistical Analysis

Experimental data are presented as the mean ± standard deviation (SD) and were analyzed by using SPSS software. Differences between groups were analyzed by using the Student *t*-test or one-way ANOVA followed by Least Significance Difference (LSD) post-hoc test. *p* < 0.05 indicated statistical significance.

Results

Autophagy is Elevated in Gastric CSCs

First, we isolated CSCs from gastric cancer MGC-803 (Figure 1A) and MKN-45 (Figure 1B) cell lines using the CD44 and CD54 surface markers. Subsequently, gastric CSC spheres were cultured using a three-dimensional cell culture system. As a result, we found that the autophagic marker LC3II was markedly enhanced in CD44⁺CD54⁺ gastric CSCs. Moreover, the ratio of LC3II/LC3I expression levels was higher in CD44⁺CD54⁺ gastric CSCs than in non-CSCs (Figure 2). These results reveal an increase in autophagy of CD44⁺CD54⁺ gastric CSCs.

Autophagy Mediates Chemoresistance of Gastric CSCs

CQ is an antimalarial drug that exhibits an inhibitory effect on autophagosome formation. To investigate the role of autophagy in chemoresistance, gastric CSCs were treated with 5-FU or CQ alone or in combination. As a result, we found that 5-FU and CQ both exhibited an inhibitory effect on the cell viability of gastric CSCs. Additionally, combination of 5-FU and CQ further suppressed the proliferation of gastric CSCs (Figure 3). These results suggest that the inhibition of autophagy enhanced the chemosensitivity of gastric CSCs.

Notch Signaling is Involved in Autophagy-Mediated Chemoresistance of Gastric CSCs

To elucidate the possible mechanism of autophagy on chemosensitivity, gastric CSCs were treated with 5-FU/CQ and inhibitors of Myc, Akt,

Wnt, NF- κ B, and Notch signaling pathways. As a result, MTT assay showed that only the addition of a Notch inhibitor decreased the numbers of gastric CSCs in the presence of 5-FU and CQ (Figure 4). These results suggest that Notch signaling is involved in autophagy-mediated chemoresistance of gastric CSCs.

Notch1 is Associated with the Chemoresistance of Gastric CSCs

Furthermore, Western blotting was used to detect the protein expression of Notch receptors and ligands in gastric CSCs treated with 5-FU alone or in combination with CQ. The combination of 5-FU and CQ markedly enhanced the protein expression of Notch1 (approximately 4.3-fold) in gastric CSCs. In addition, administration of 5-FU alone increased the protein levels of Notch1 by approximately 1.5-fold in gastric CSCs (Figure 5). Taken together, these data demonstrate that autophagy mediated chemoresistance via Notch1 in gastric CSCs.

Discussion

Gastric cancer is the second leading cause of cancer-related deaths worldwide. Although improvements in the quality of combined surgery and chemotherapy treatments have been made, prognosis of gastric cancer is poor, especially in terms of tackling advanced and disseminated gastric tumors¹⁷. The main cause of treatment failure for gastric cancer is the development of drug resistance to the chemotherapeutic agents, which currently represent the primary treatment options¹⁸. Accumulating evidence^{4,5} demonstrates the existence of CSCs in solid tumors of a wide variety of organs. CD44, a transmembrane glycoprotein, is a well-known CSC marker in several cancers, including prostate, breast, gastric, and colon cancers¹⁹. CD54, also known as ICAM-1, is widely expressed in stromal, tumor, and immune cells²⁰. Chen et al⁹ previously demonstrated that the adhesion molecules CD44 and CD54 were positively expressed by most cells in gastric

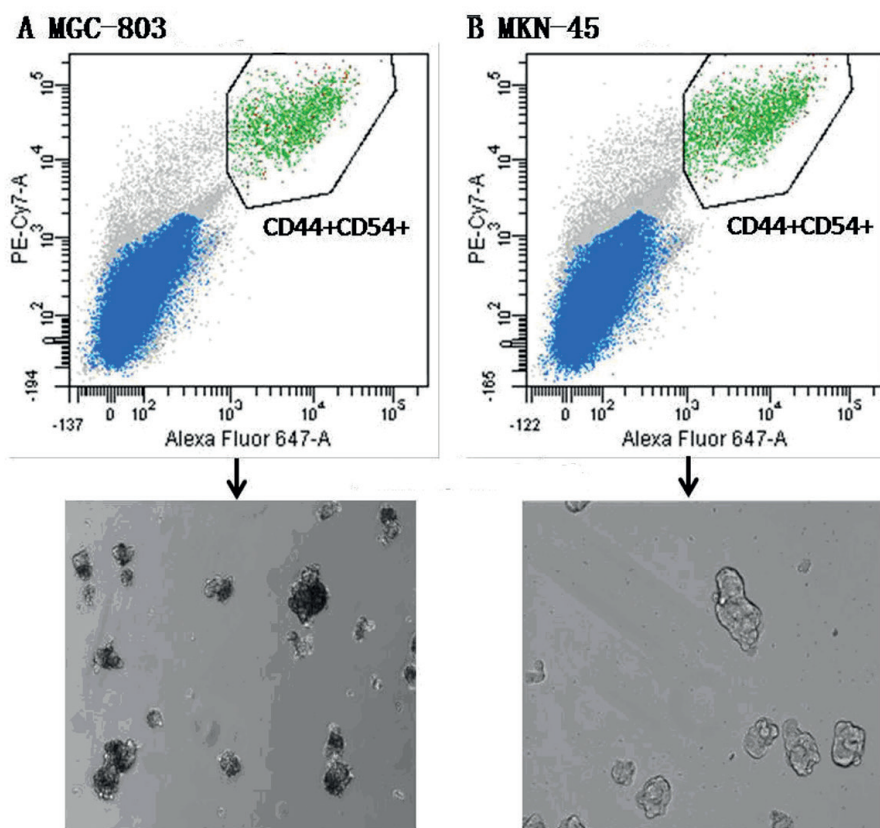


Figure 1. Isolation and culture of CD44⁺CD54⁺ stem cells from gastric cancer cells. CD44⁺CD54⁺ stem cells were isolated from the gastric cancer cell lines MGC-803 (A) and MKN-45 (B) and cultured in a three-dimensional cell culture system.

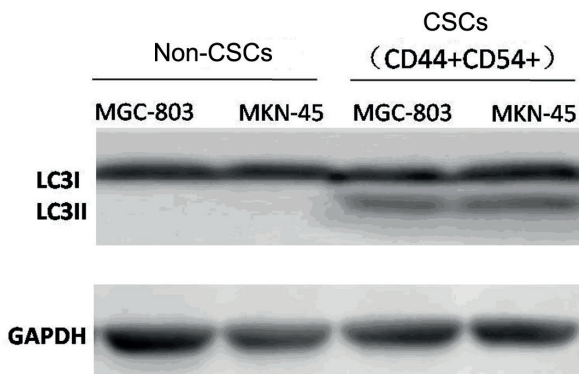


Figure 2. Autophagy levels in CD44⁺CD54⁺ gastric CSCs. Western blot was used to detect the expression of the autophagy markers LC3I/II. GAPDH was used as an internal control.

tumorigenic spheres and human gastric cancer tissues. These data suggest that CD44 and CD54 can serve as surface markers for gastric CSCs. In the present study, we successfully isolated CD44⁺CD54⁺ stem cells from gastric cancer cell lines and cultured these CSCs in a three-dimensional cell culture system.

Several mechanisms are responsible for drug resistance in human cancers, including activation of Wnt, sonic hedgehog, and Notch signaling pathways^{21,22}. Moreover, a recent investigation²³ demonstrated the involvement of the autophagy genes in many aspects of tissue homeostasis, organelle turnover, carcinogenesis, and neurodegenerative and cardiovascular diseases. Our current study reveals an increase in autophagic marker LC3II level, as well as LC3II/LC3I ratio, in CD44⁺CD54⁺ gastric CSCs compared with that

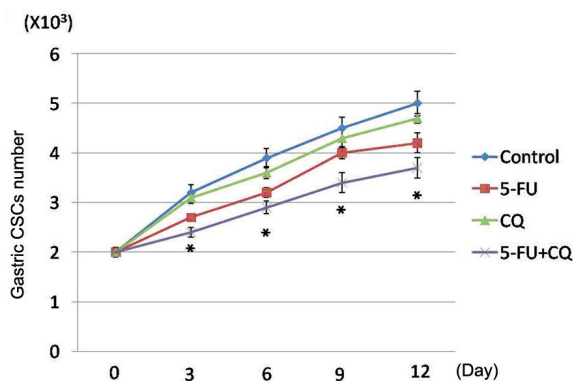


Figure 3. Effect of autophagy inhibition on chemoresistance of gastric CSCs. Gastric cancer stem cells were treated with 5-FU or autophagy inhibitor CQ alone or in combination. The number of gastric CSCs was counted at different time points (3, 6, 9, and 12 days). * $p < 0.05$.

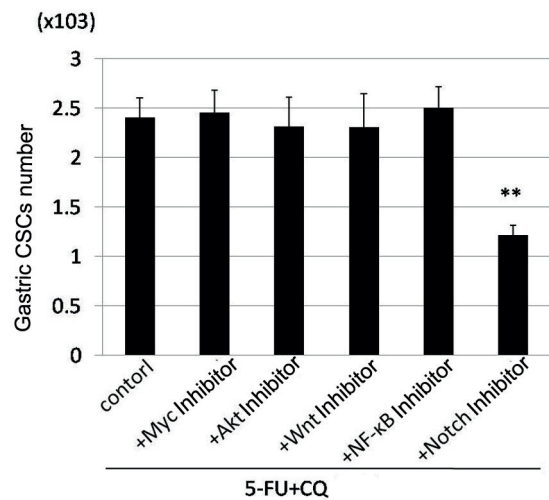


Figure 4. Role of Notch signaling in autophagy-mediated chemoresistance of gastric CSCs. Gastric CSCs were treated with 5-FU/CQ and inhibitors of Myc, Akt, Wnt, NF-κB, and Notch signaling pathways. The number of gastric CSCs was counted in the different groups. ** $p < 0.01$.

in non-CSCs, suggesting that the autophagic activity was enhanced in gastric CSCs. In addition, gastric CSCs were treated with 5-FU or CQ alone

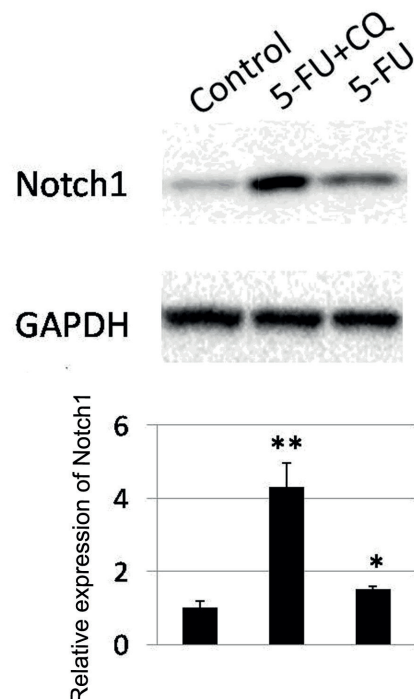


Figure 5. Notch1 is associated with the chemoresistance of gastric CSCs. Gastric CSCs were treated with 5-FU alone or in combination with CQ. Western blotting was used to detect the protein expression of Notch1. The protein bands were normalized to GAPDH. * $p < 0.05$, ** $p < 0.01$

or in combination to evaluate the role of autophagy in drug resistance. We found that 5-FU and CQ both decreased the viability of gastric CSCs and that their combination further enhanced such inhibitory effects. These findings suggest that autophagy contributed to the chemoresistance of gastric CSCs and that the inhibition of autophagy may be considered as a novel therapeutic tool for gastric cancer therapy.

Notch signaling can manipulate physiological and pathological processes, including stem cell renewal and differentiation, tumorigenesis, immune response, and cardiovascular diseases²⁴. Increasing evidence²⁵ reveals that the Notch signaling network is frequently dysregulated in various human malignancies, such as breast, gastric, colon, and liver cancers. In our study, inhibition of Notch signaling suppressed the cell viability of gastric CSCs in the presence of 5-FU and CQ. In addition, 5-FU alone or in combination with CQ significantly increased Notch1 expression in gastric CSCs. These data suggest that Notch signaling is involved in autophagy-mediated chemoresistance.

Conclusions

We successfully isolated CSCs from gastric cancer cells by using CD44 and CD54 surface markers. Furthermore, we found that autophagy mediated drug resistance via the Notch signaling pathway in gastric CSCs, suggesting that manipulation of autophagy should be developed as a novel tool for gastric cancer therapy.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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