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Indentification of breast cancer subtypes sensitive to HCQ-induced autophagy inhibition

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ABSTRACT

Background: Breast cancer is the most frequent carcinoma in females, which could be classified to 4 subtypes and the current treatment is still far from satisfactory. In this study, we explored the effects of autophagy inhibition on certain subtypes of breast cancer and the molecular mechanism underlying the different response for breast cancer subtypes initially.

Methods: Autophagy inhibitor hydroxychloroquine (HCQ) was used to identify the sensitivity of breast cancer subtypes to autophagy inhibition in the present study. Cell proliferation and cell invasion were assessed by Cell Counting Kit-8 assay (CCK-8) and transwell assay, respectively. Immunofluorescence staining and western blotting were applied to evaluate cell autophagy. In addition, levels of Ras/Raf/ERK signaling pathway were evaluated by western blotting.

Results: Our results showed that HCQ treatment suppressed breast cancer cell proliferation and migration in especially SUM-190 cells, which was most sensitive. Furthermore, HCQ inhibited cell autophagy in breast cancer cells by regulating levels of p62, LC3-I and LC3-II. Moreover, the expression of Ras was significant lower than other breast cancer cells. HCQ treatment markedly inhibited the activation of Ras/Raf/ERK signaling in SUM190 cells.

Conclusion: To conclude, basal-like breast cancer represented by SUM-190 cells may be most sensitive to HCQ induced autophagy inhibition and the mechanism might be relative to Ras/Raf/ERK signaling pathway.

1. Introduction

Breast cancer is the most frequent carcinoma in females, diagnosed in 1.4 million women in the US every year [1] and has been the most common cause for cancer mortality in women [2]. Even with new progress in screening, diagnostics and surgery extra, there are still a lot left to be desired. Over the past decade, increasing molecular and genetic knowledge [3–5] allow us to classify breast cancer into at least four different molecular types, namely luminal A, luminal B, HER2 and basal-like [6]. Each subtype shows prognosis and treatment response in a intrinsically different manner [7]. Therefore, it's necessary to reach the level of molecular subtype when seeking the possible novel treatment for breast cancer.

The term autophagy is used to describe lysosomal degradation, or just eating (phagy) part of the cell itself (auto).There are several forms of autophagy, among which macroautophagy (hereafter referred to as autophagy) indicates a process of cytoplasmic components engulfed in autophagosome (a double membrane structure) to be delivered to degrade in the lysosome eventually [8]. Recently, a tremendous effort has been made to understand the role of autophagy in different cancers, including breast cancer and autophagy is found to exert either tumor suppressive [9,10] or promoting [11,12] functions. In general, most evidences support that autophagy could be a tumor promoter by enabling tumor cell survival. Thus, autophagy inhibition might be beneficial for cancer prevention. Notably, numerous clinical trials targeting autophagy by non-specific inhibitors chloroquine (CQ) or hydroxychloroquine (HCQ) in various cancers are in progress, and the preliminary results suggested the need for reliable patient selection criteria [13,14].

This study aims to determine whether certain subtypes of breast cancer are sensitive to autophagy-related inhibition strategies, with the longer term purpose to achieve better patient selection for relevant treatments. In addition, we'd like to search the molecular mechanism underlying the different response for breast cancer subtypes initially.

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Table 1
Cell lines and corresponding molecular subtypes in breast cancer.

luminal A	luminal B	HER2 positive	basal-like
MCF-7	BT474	MDA-453	SUM190

2. Materials and methods

2.1. Cell culture

Four breast cancer cell lines MCF-7, BT474, MDA-453 and SUM190 (ATCC, Manassas, VA, USA) were included in this study. All cell lines were cultured in DMEM (Gibco, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS, Sigma, F6178) at 37 °C and 5% CO_2.

2.2. Cell counting Kit-8 assay

Cells were cultured at a density of 5×10^3 cells/well in 96-well plates, then treated with 5-FU and/or HCQ for 24 h. Each group was repeated six times. After the exposure period, 10 μl CCK-8 (Beyotime) reagent was added to each well. Absorbance was measured at 450 nm using microplate spectrophotometry. All assays were performed in triplicate.

2.3. Transwell assay

Cells were serum-starved for 24 h and plated at the density of 5×10^5 cells per well in the serum-free medium. DMEM containing 10% FBS (Thermo Fisher) was added in the bottom chamber (8-μm pore size, BD Pharmingen, San Diego, CA, USA). After incubation for 24 h, remove non-migrating cells with a cotton swab. Cells migrated through the membrane were fixed with 4% formaldehyde (Sigma) and stained with crystal violet staining solutions (Sigma).

2.4. Immunofluorescence assay

Cells were plated on coverslips until the confluence reached to 70%.Then the cells were washed three times with cold PBS after treatments and fixed in 4% paraformaldehyde for 20 min at room temperature and washed three times by PBS. After that, cells were permeabilized with 200 μg/ml digitonin (Invitrogen) for 15 min and incubated with primary anti-LC3B antibody (1:2000, abcam) overnight at 4 °C. After washing with PBS for three times, cells were then incubated with secondary antibodies for 2 h, washed three times in PBS and stained with DAPI (Vector) for 5 min. Images were acquired by

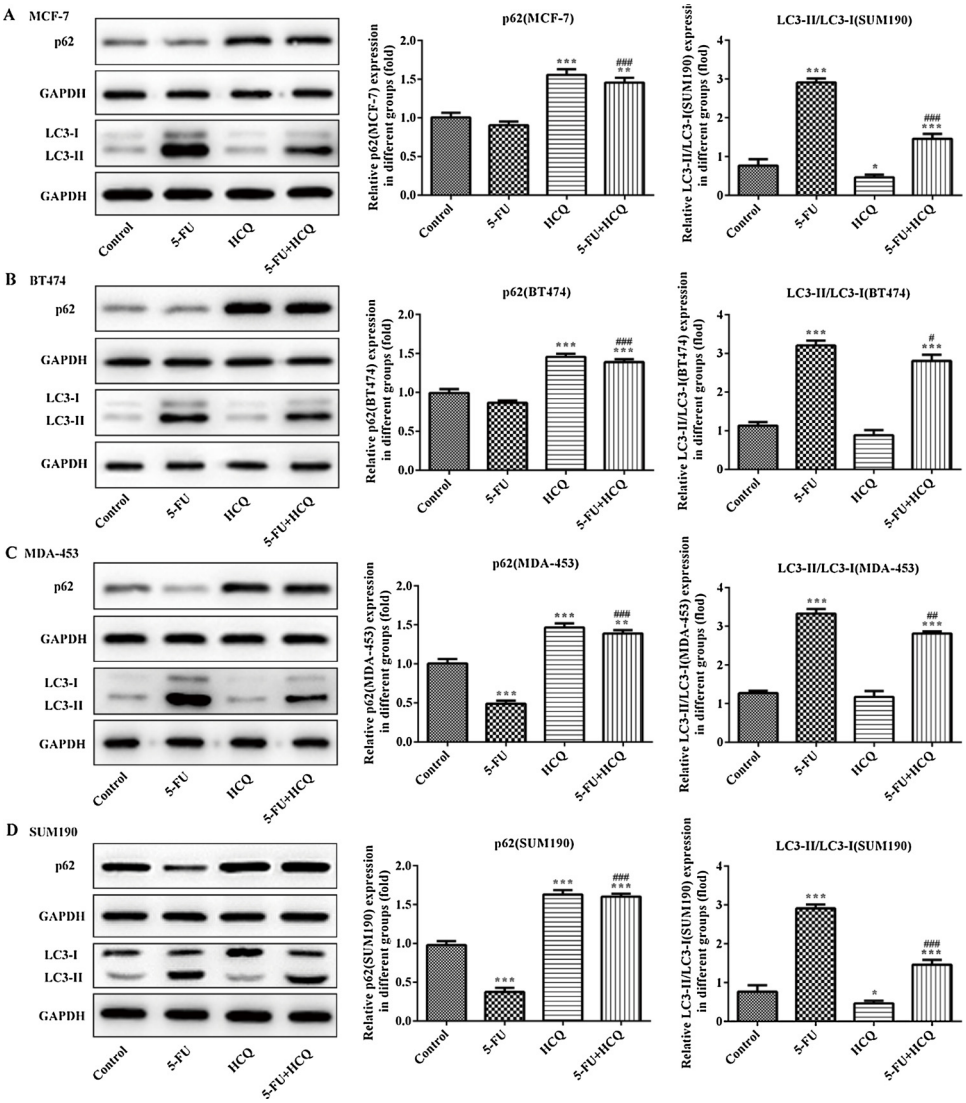

Fig. 1. Effects of 5-FU and HCQ on the protein expressions of p62 and LC3II/LC3I in MCF-7 (A), BT474 (B), MDA-453 (C) and SUM190 (D) cell lines. Data are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$vs. 5-FU group.

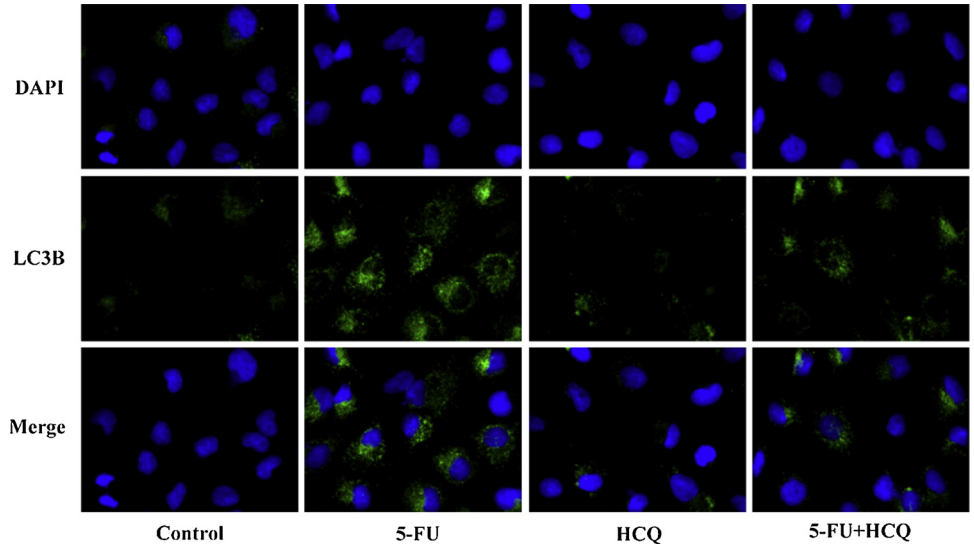

Fig. 2. Immunofluorescence staining of LC3B in SUM190 cells treated with 5-FU or/and HCQ. Image magnification: 200 × .

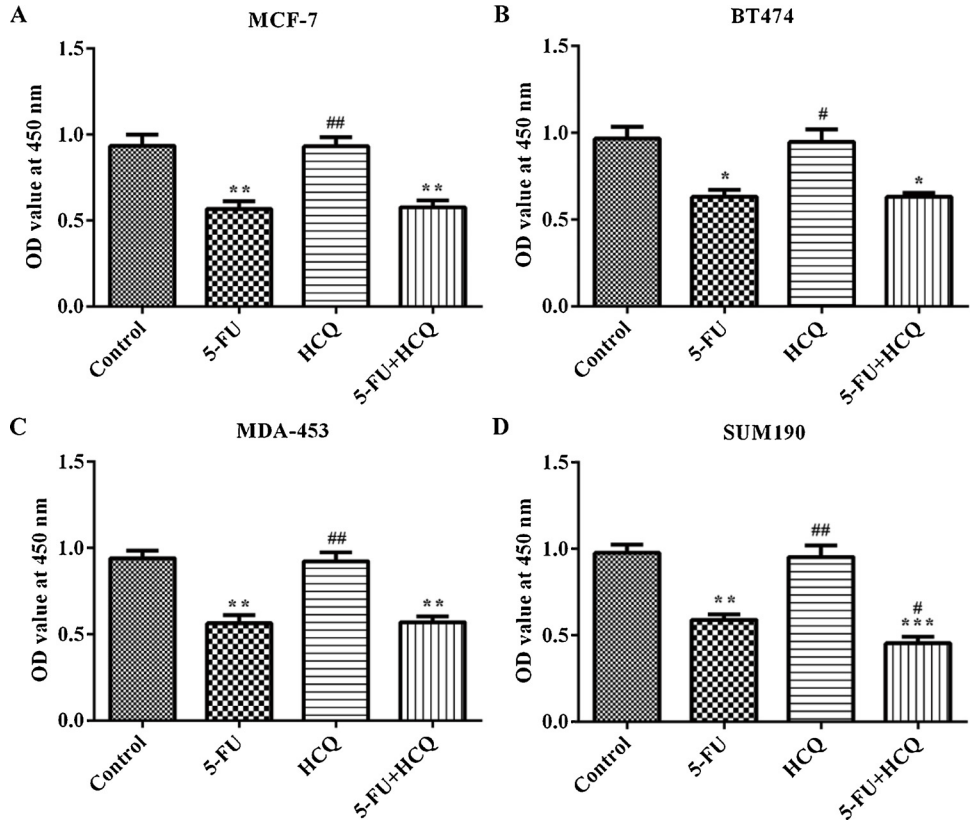

Fig. 3. Cell viability in MCF-7 (A), BT474 (B), MDA-453 (C) and SUM190 (D) cell lines treated with 5-FU or/and HCQ was detected by CCK-8 assay. Data are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$vs. 5-FU group.

confocal microscopy Zeiss LSM-700 (Zeiss).

2.5. Western blot

Total protein was extracted with RIPA buffer and a BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to measure the protein concentration. 10% SDS-PAGE gel was applied and protein strips were then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocked by 5% BSA, the membranes were incubated with primary antibodies (p62, 5 μg/ml, ab56416; LC3, 1:1000, ab244212; Ras, 1:1000, ab108602; Raf-1, 1:1000, ab230850; ERK1/2 1:5000, ab79853; GAPDH, 1:5000, ab9482) respectively at 4 °C overnight. Afterwards, corresponding HRP-conjugated secondary antibodies (1:5000, Abcam) were used and protein blots were detected by using the ECL Detection System (Bio-Rad Universal Hood II system).

2.6. Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics v20.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SD. The

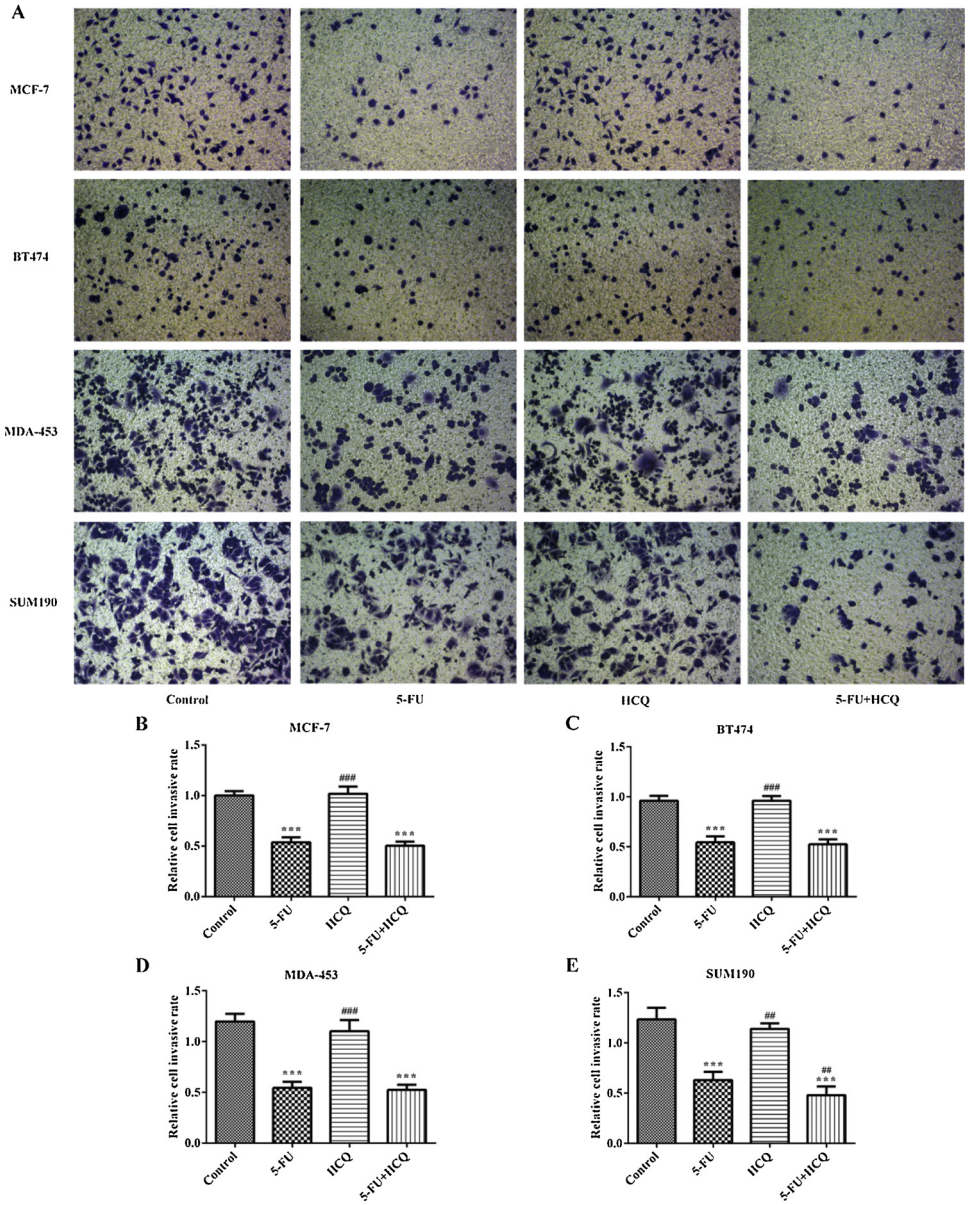

Fig. 4. Effects of 5-FU and HCQ on cell invasion in MCF-7, BT474, MDA-453 and SUM190 cell lines. Cell invasion (A) and the relative cell invasive rates (B) of MCF-7, BT474, MDA-453 and SUM190 cells were evaluated by transwell assay. Image magnification: 100 × . Data are expressed as mean ± SD, n = 3. $^{***}P < 0.001$ vs. control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$vs. 5-FU group.

data significance was measured by Student's *t*-test and one-way analysis of variance. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. HCQ inhibits 5-FU induced autophagy in breast cancer cells

Four cell lines of MCF-7, BT474, MDA-453 and SUM190 were chosen as representatives of breast cancer molecular subtypes in our study (Table 1). LC3 is the mammalian homolog of Atg8 and could be cleaved and conjugated to phosphatidylethanolamine during autophagy. As LC3 could be conjugated and degraded during autophagy process into LC3II, LC3II accumulation caused by lysosomal inhibition can be applied as a measure of basal autophagic flux [15,16]. p62, which contains an LC3-interacting region, also plays a critical role in both autophagy and apoptosis [17]. Therefore, autophagy level was evaluated by the expression of LC3 and p62. As shown in Fig. 1A, HCQ treatment and 5FU + HCQ treatment significantly promoted p62 protein level when compared with the control and 5-FU + HCQ treatment showed a significant increase of p62 protein level compared to 5-FU treatment. Furthermore, the levels of LC3II/LC3I in 5-FU treatment and 5FU + HCQ treatment were elevated when compared with the control while that in 5-FU + HCQ treatment was significantly decreased compared to 5-FU treatment. In other cells of BT474, MDA-453 and SUM190, similar trends were observed respectively exhibited in Fig. 1B–D. However, the level of protein p62 showed a considerably decreased in 5-FU treatment in MDA-453 cells and SUM190 cells compared to the control and the expression of LC3II/LC3I in HCQ treatment in SUM190 cells was notably decreased compared to the control.

To further study the autophagy-inhibited effects of HCQ on breast cancer cells, immunofluorescence assay was performed. The results showed that the expression level of LC3B was extremely increased in 5-FU treatment and HCQ treatment reversed 5-FU-induced autophagy (Fig. 2). To sum up, our results revealed that in the present study HCQ

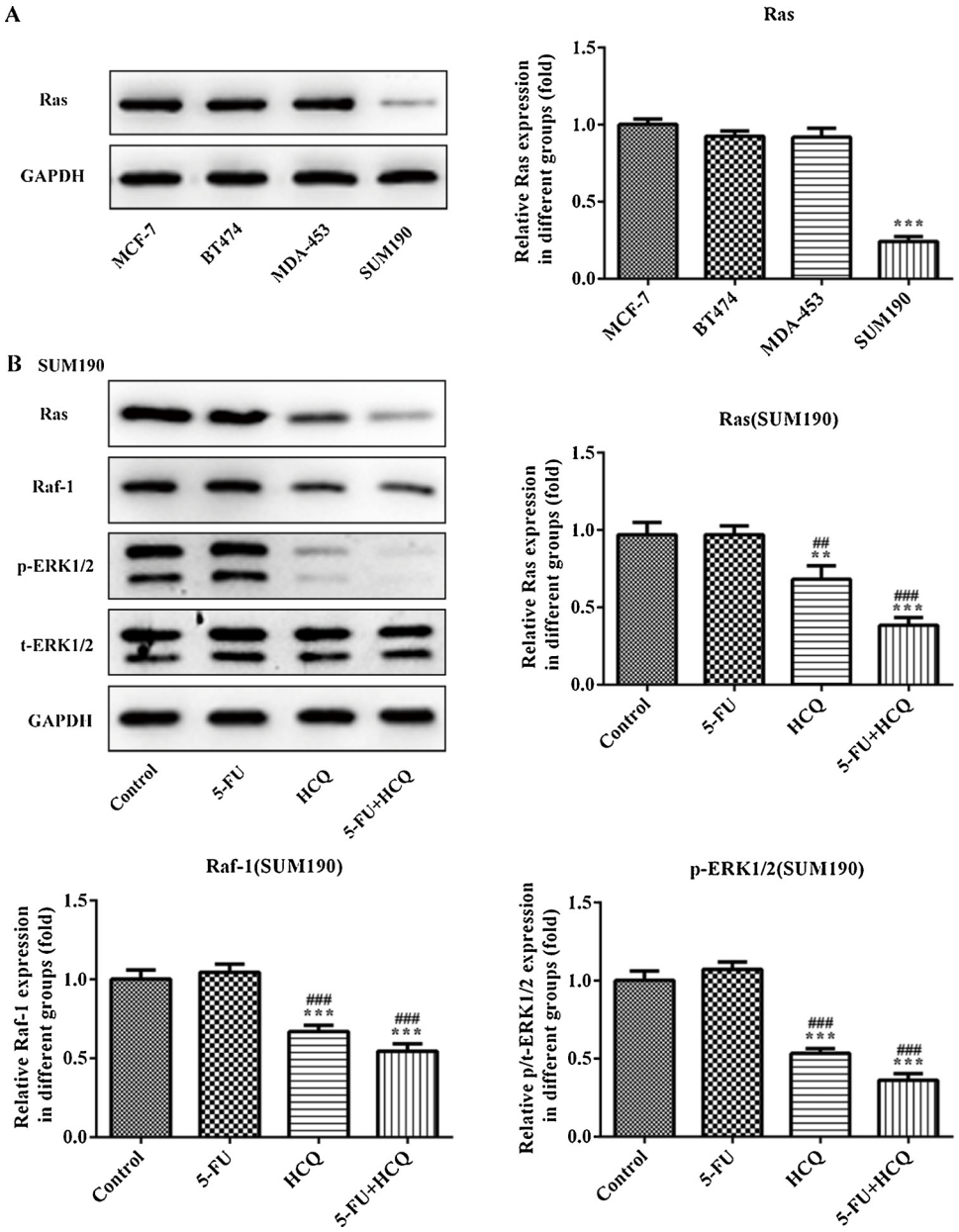

Fig. 5. Effects of 5-FU and HCQ on protein expressions of Ras, Raf-1 and p-ERK1/2 in MCF-7, BT474, MDA-453 and SUM190 cell lines. (A) The protein level of Ras in MCF-7, BT474, MDA-453 and SUM190 cell lines was determined by western blotting. (B) The protein level of Ras, Raf-1 and p-ERK1/2 in SUM190 cells treated with 5-FU or/and HCQ were determined by western blotting. Data are expressed as mean ± SD, n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$vs. 5-FU group.

effectively inhibited 5-FU induced autophagy in breast cancer cells, with the best autophagy-inhibited effect in SUM190 when co-treated with 5FU and HCQ.

3.2. SUM190 cells are most sensitive to HCQ-induced autophagy inhibition

Then the viability of different breast cancer cells with HCQ or/and 5-FU were tested by CCK-8 assay. In Fig. 3A, MCF-7 cells showed a normal viability in control and HCQ-treated group while 5-FU with or without HCQ incubation markedly suppressed the cell viability. There was no significant difference between 5-FU and 5-FU + HCQ group in term of cell viability. Likewise, BT474 and MDA-453 cells showed similar phenomenon in Fig. 3B and C. However, for SUM190 cells 5-FU and HCQ co-treatment significantly decreased cell viability compared with 5-FU incubation alone as shown in Fig. 3D. In addition, cell invasive ability was measured via transwell assay. In Fig. 4A-D, 5-FU and 5-FU + HCQ dramatically reduced cell invasive ability in MCF-7, BT474 and MDA-453 cells. Furthermore, 5-FU and 5-FU + HCQ treatment reduced cell invasive ability compared to the control and HCQ + 5-FU treatment significantly reduced cell invasive ability compared to 5-FU treatment in SUM190 cells (Fig. 4E). These results demonstrated that among cell lines used in the study, SUM190 cells were most sensitive to HCQ-induced autophagy inhibition.

3.3. HCQ regulates autophagy in SUM190 cells via Ras/Raf/ERK signaling pathway

We also further researched the possible mechanism underlying the high sensitivity of SUM190 cells towards HCQ, Previous reports indicated that Ras/Raf/ERK signaling pathway may contribute to autophagy inhibition sensitivity in breast cancer [18]. Therefore, we continued to evaluate the protein levels involved in Ras/Raf/ERK

signaling in breast cancer cell lines. As expectation, it was observed that SUM190 cells showed an obviously higher level of Ras than other cell lines when treated with HCQ (Fig. 5A). Furthermore, the expressions of Ras, Raf-1 and p-ERK1/2 in HCQ and 5-FU + HCQ treatment were considerably down-regulated compared to the control in SUM190 cells. In addition, 5-FU + HCQ treatment significantly reduced the levels of Ras, Raf-1 and p-ERK1/2 when compared to the 5-FU treatment (Fig. 5B). The results showed that HCQ exerts autophagy inhibition in SUM190 cells at least partly via Ras/Raf/ERK signaling pathway.

4. Discussion

Breast cancer has been threatening human health for a long time and multiple researches have been carried out to investigate the underlying mechanism of breast cancer [19,20]. During exploration, it was found that breast cancer contains different molecular types, namely luminal A, luminal B, HER2 and basal-like type with distinguishing features [21–23]. According to current guidelines, chemotherapy remains one of the most important treatments for breast cancer. However, cell tolerance weakens the efficacy of chemotherapy seriously [24]. It is noteworthy that different molecular subtypes of breast cancer show different response to chemotherapy, which requires us to push related researches deeper to the level of molecular subtypes [25,26]. In the present study, we aimed to investigate how HCQ induced autophagy inhibition may affect 5-FU sensitivity in breast cancer cells. Breast cancer cell lines MCF-7, BT474, MDA-453 and SUM190 representing luminal A, luminal B, HER2 and basal-like type respectively were chosen for the subsequent experiments [27]. Our findings in the current study can be listed as following: 1) HCQ treatment could inhibit autophagy process in MCF-7, BT474, MDA-453 and SUM190 cells. 2) SUM 190 cells were most sensitive to HCQ induced autophagy inhibition compared with other cell lines. 3) HCQ may regulate autophagy in SUM190 cells via Ras/Raf/ERK signaling pathway.

Autophagy was previously suggested to play a role in tumor progression [28] and may be potential to inhibit breast cancer development [29]. Kazuhito Sasaki and colleagues [30] demonstrated Chloroquine could potentiate the anti-cancer effect of 5-FU on colon cancer cells. LC3B was found to highly express in breast cancer compared with normal tissues and increasing threshold of LC3B activation forces the breast cancer cells undergo growth inhibition [31]. Most researchers advocate that diverse functions of autophagy may depend on the specific cell types and more studies are needed to investigate the molecular subtype difference [32]. In this study, we found in all 4 cell lines of breast cancer, HCQ induced significant autophagy inhibition and HCQ + 5-FU co-treatment made stronger inhibitory effect on cell proliferation than 5-FU alone in SUM190 cells. SUM190, which belongs to the basal-like subtype of breast cancer, is most sensitive to HCQ induced autophagy inhibition according to our results. These data demonstrated that autophagy-inhibition function of HCQ varied in different molecular subtypes in breast cancer and it was suggested that basal-like breast cancer might be more suitable for autophagy therapy in clinical.

Then we continued to investigate the possible mechanism underlying the sensitivity difference in breast cancer cell lines. Ras/Raf/ERK pathway is well-known to play a vital part in various physiological processes including inflammation, tumor development, especially autophagy and apoptosis [33]. Bartholomeusz et al. found that

PEA-15 served as tumor inhibitor via the induction of autophagy involving activation of the ERK1/2 pathway [34]. Liang et al. showed that Polygonatum odoratum lectin induces MCF-7 cell apoptosis and autophagy via targeting EGFR-mediated Ras/Raf/MEK/ERK signaling pathway [35]. In addition, Wang et al. discovered that bicyclol inhibited cell proliferative activity and induced autophagy in malignant human hepatoma cells via regulating the PI3K/AKT pathway and the Ras/Raf/MEK/ERK pathway [36]. Therefore, we tried to explore whether Ras/Raf/ERK pathway plays a part in cell sensitivity difference towards HCQ in breast cancer cells. As shown in Fig. 5, Ras showed a significantly lower level in SUM190 cells than other cell lines which is consistent with the results of highest sensitivity of SUM190 cells. Additionally,

Ras, Raf-1 and p-ERK1/2 expression levels decreased when treated with HCQ. Those data showed that Ras/Raf/ERK signaling pathway may take part in regulation of autophagy inhibition from HCQ in SUM190 cells. However, further research is necessary to obtain specific mechanism how the Ras/Raf/ERK signaling pathway regulates autophagy.

Taken together, our results demonstrated that HCQ induced autophagy inhibition may help promote cell sensitivity to chemotherapy especially in SUM 190 cells and this effect was at least partially mediated by inhibition of Ras/Raf/ERK signaling pathway. The present study indicates that basal-like breast cancer is likely to be the most suitable subtype for autophagy inhibition therapy for breast cancer patients.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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