Gynecologic Oncology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Expression and role of autophagy-associated p62 (SQSTM1) in multidrug resistant ovarian cancer☆

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HIGHLIGHTS

- Expression of p62 is higher in primary ovarian cancer tissue than in patient-matched metastasis and recurrent tumors.
- Higher expression of p62 is associated with favorable prognosis in both overall survival and disease-free survival.
- Drug resistant cancer cells exhibit a high level of autophagic activity.
- Inhibition of autophagy enhances paclitaxel sensitivity in drug resistant ovarian cancer cells.

ARTICLE INFO

Article history:
Received 6 February 2018
Received in revised form 9 April 2018
Accepted 12 April 2018
Available online xxxx

Keywords:
Ovarian cancer
Autophagy
p62
Drug resistance
Paclitaxel

ABSTRACT

Objective. Multidrug resistance is the major cause of treatment failure in ovarian cancer. p62 (SQSTM1) is a multifunctional protein involved in multiple cellular processes including proliferation, drug sensitivity and autophagy-associated cancer cell growth. However, the role of p62 in drug resistance remains controversial.

Methods. In this study, we examined p62 expression by immunohistochemistry in a unique ovarian cancer tissue microarray (TMA), which was constructed with paired primary, metastatic, and recurrent tumor tissues. The expression levels of p62 and autophagy related proteins were evaluated in two panels of human cancer cell lines by western blot. Cell viabilities were determined by MTT assay after exposure ovarian cancer cells to different concentrations of paclitaxel alone or in combination with autophagy inhibitors.

Results. Both the metastatic and recurrent tumor tissues expressed less p62 than the patient-matched primary tumor. A significant inverse correlation has been found between p62 expression and both the disease-free survival and overall survival. Additionally, multidrug resistant cancer cell lines expressed lower levels of p62 as compared with their parental drug sensitive cell lines. Importantly, inhibition of autophagy enhanced paclitaxel sensitivity in drug resistant ovarian cancer cells. Furthermore, the wound healing assay exhibited that the inhibition of autophagy significantly decreased resistant ovarian cancer cell migration *in vitro*.

Conclusion. Our findings highlight the potential of p62 as a new prognostic marker for ovarian cancer patients and p62's associated autophagy pathway may be a promising therapeutic target to prevent metastasis, recurrence and to reverse drug resistance in ovarian cancer.

1. Introduction

Ovarian cancer is the most lethal gynecological malignancy, and ranks as the fifth leading cause of cancer death among women. The American Cancer Society estimates that about 22,280 new ovarian cancer cases and 14,240 deaths will occur in the United States in 2016 [1]. Most ovarian cancer patients are asymptomatic and thus approximately 60% of patients have advanced stage III and IV at diagnosis. Despite ongoing advances in treatment therapies, the overall 5-year survival rate of ovarian cancer patients increased slightly from 36% in 1975 to 46%

☆ This work has been selected for presentation in 2018 AACR Annual Meeting, Chicago, USA.

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https://doi.org/10.1016/j.ygyno.2018.04.557
0090-8258/

in 2011 [1,2]. The current standard treatment for advanced ovarian cancer includes primary cytoreductive surgery followed by adjuvant combination chemotherapy with paclitaxel and platinum [3,4]. Over 80% of tumors are initially sensitive to standard chemotherapy, but acquire broad cross resistance (multidrug resistance, MDR) that can include a variety of structurally and functionally unrelated chemotherapeutic agents [5]. MDR severely limits the ultimate success of chemotherapy treatment, leading to patient relapse or even death due to drug resistant/metastatic disease [6].

Mechanisms of MDR in ovarian cancer have been associated with several characteristics including: overexpression of a plasma membrane glycoprotein (Pgp), changes in specific proteins targeted by chemotherapy drugs, and alterations in the apoptotic threshold [7–9]. However, the evidence linking these mechanisms to acquired drug resistance in tumors in clinically relevant MDR is lacking. More recent studies have revealed an association between drug resistance and autophagy [10,11]. Autophagy is a cellular mechanism that targets unnecessary or dysfunctional cellular organelles and proteins for degradation in order to overcome stress [12]. This dynamic process includes several key steps: induction, vesicle nucleation and elongation as well as the formation of autophagosomes and autolysosomes [13,14]. Autophagy pathways are activated as a protective mechanism to mediate the acquired MDR phenotype of some cancer cells during chemotherapy [10,15]. Moreover, increasing evidence suggests that the inhibition of autophagy can augment cytotoxicity and restore chemosensitivity in combination with conventional anticancer drugs [16,17]. Autophagy inhibitors can be divided into two categories: upstream inhibitors and downstream inhibitors. 3-MA and Wortmannin belong to the upstream autophagy inhibitors, which block the formation of autophagosomes by suppressing class III phosphatidylinositol 3-kinase (PI3k). Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are the downstream autophagy inhibitors, which suppress autophagosome and lysosome fusion, which form autophagolysosomes that degrade the autophagic cargo inside of autolysosomes [18].

A series of autophagy related proteins, particularly microtubule-associated protein 1 light chain 3 (LC3) and Beclin-1, have been used as indicators of autophagy [19,20]. To date, only LC3 is known to exist on the membrane of autophagosomes, so the amount of LC3 usually correlates well with the extent of autophagosome formation [21]. It should be noted that the accumulation of autophagosomes may represent either autophagy induction or a block of autophagosomal maturation [19]. Therefore, the numbers of autophagosomes are insufficient to comprehensively assess the autophagic activity and other approaches that are required to distinguish between induction or suppression of autophagy. Several specific substrates are efficiently degraded by autophagy, of which the well-characterized receptor is p62 (also known as SQSTM1/sequestome 1) [19]. p62 is a vital indicator of autophagic flux, which selectively incorporates into autophagosomes by directly binding to the LC3 on autophagic membranes for subsequent degradation in autolysosomes [22,23]. The term “autophagic flux” refers to the dynamic process of autophagy activity including: autophagosome synthesis, transportation of autophagic substrates to the lysosome, and subsequent degradation inside of the lysosome. As an indicator of autophagy, autophagic flux is more reliable than the number of autophagosomes [19]. Inhibition of autophagy correlates with increased levels of p62 [24]. Therefore, the total cellular expression level of p62 inversely reflects autophagic activity. However, the role of p62 in paclitaxel resistance in ovarian cancer has not been determined. In the present study, we evaluated the expression of p62 in ovarian cancer tissues and cell lines. Our findings showed that p62 was significantly decreased in metastatic and recurrent ovarian cancer tissues and drug resistance cell lines. These results suggest that autophagy plays a key role in the emergence of drug resistance, p62 is a potential predictor of MDR in ovarian cancer. Our study suggests that the targeting of the autophagy pathway can be a potential strategy to reverse drug resistance.

2. Materials and methods

2.1. Ovarian cancer tissue microarray (TMA)

The archived, formalin-fixed, paraffin-embedded ovarian cancer TMA was used in the present study, which was constructed by the Tissue Microarray Core and Imaging Core at the Dana-Farber/Harvard Cancer Center [25]. Specimens were obtained from 26 individual ovarian cancer patients during their treatment at Massachusetts General Hospital. Each of these 26 patients' tumor tissue blocks were composed of: 1) a primary tumor, 2) a synchronous metastasis obtained at the time of the primary surgery, and 3) a metachronous recurrence from the same patient collected at the time of tumor recurrence after combination chemotherapy with paclitaxel and platinum. Slides stained with hematoxylin and eosin (HE) from each tissue block were reviewed by a senior consultant pathologist, together with a pathology technician to obtain representative triplicate 0.5-mm-diameter core biopsies of primary, metastasis, and recurrent ovarian cancer tumors (absence of necrosis, poorly differentiated tumor areas). Tumor staging was assessed in accordance with the Federation International of Gynecology and Obstetrics (FIGO) and TNM staging system. Histological subtype and tumor grade was classified according to the World Health Organization (WHO) 2014 guidelines. Clinical information was also collected, including: whether ascites was present at surgery, patient status at last follow-up, disease-free survival (DFS) defined as the interval between the date of diagnosis and the date of recurrence, and lastly overall survival (OS) defined as the interval between the date of surgery to last follow-up or death. The range of DFS is between 5.3 and 53.3 months. Moreover, the shortest OS of a patient is 12 months while the longest follow-up of a living patient is 162.3 months. Acquisition of tissue samples and clinical information was approved by the Institutional Review Board at Massachusetts General Hospital.

2.2. Immunohistochemistry

To further determine the expression level of p62 in ovarian cancer TMA, an Immunohistochemistry assay was performed as previously described [25]. The complete experimental details are presented in the Supplementary Materials.

2.3. Cell lines and reagents

Previously, we characterized and described the drug sensitive ovarian cancer cell line SKOV3 and osteosarcoma cell line U2OS, and its MDR derivatives SKOV3TR and U2OSR2 that were used in this study [26,27]. Both SKOV3TR and U2OSR2 were selected from their respective parental cell lines post exposure to stepwise increases in paclitaxel concentrations. At 37 °C, all cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) with an atmosphere of 5% CO2 and 95% air humidified. The cell cultures were routinely passaged every 3–5 days. Autophagy inhibitors 3-MA and HCQ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paclitaxel was obtained from the pharmacy at the Massachusetts General Hospital. p62 is a mouse monoclonal antibody raised against SQSTM1 amino acids 151–440 of human origin, purchased from Santa Cruz Biotechnology, INC. The autophagy antibody sampler kit and monoclonal rabbit anti-human Pgp antibody were acquired from Cell Signaling Technology (Danvers, MA, USA). Lipofectamine®3000 was purchased from Life Technologies (Grand Island, NY, USA).

2.4. Western blot

Western blot was performed as previously described to assess the expression level of p62 and other autophagy related proteins, LC3,

Please cite this article as: J. Wang, et al., Expression and role of autophagy-associated p62 (SQSTM1) in multidrug resistant ovarian cancer, Gynecol Oncol (2018), https://doi.org/10.1016/j.ygyno.2018.04.557