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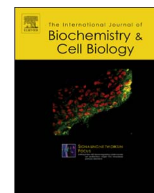

Hexokinase 2 confers resistance to cisplatin in ovarian cancer cells by enhancing cisplatin-induced autophagy

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ABSTRACT

The high mortality rate of ovarian cancer is connected with the development of acquired resistance to multiple cancer drugs, especially cisplatin. Activation of cytoprotective autophagy has been implicated as a contributing mechanism for acquired cisplatin resistance in ovarian cancer cells. Hexokinase 2 (HK2) phosphorylates glucose to generate glucose-6-phosphate, the rate-limiting step in glycolysis. Higher HK2 expression has been associated with chemoresistance in ovarian cancer. However, whether HK2 functionally contributes to cisplatin resistance in ovarian cancer is unclear. In this study, we investigated the role of HK2 in regulating ovarian cancer cisplatin resistance. Increased HK2 levels were detected in drug-resistant human ovarian cancer cells and tissues. Cisplatin downregulated HK2 in cisplatin-sensitive but not in resistant ovarian cancer cells. HK2 knockdown sensitized resistant ovarian cancer cells to cisplatin-induced cell death and apoptosis. Conversely, HK2 overexpression in cisplatin-sensitive cells induced cisplatin resistance. Mechanistically, cisplatin increased ERK1/2 phosphorylation as well as autophagic activity. Blocking autophagy with the autophagy inhibitor 3-MA sensitized resistant ovarian cancer cells to cisplatin. HK2 overexpression enhanced cisplatin-induced ERK1/2 phosphorylation and autophagy while HK2 knockdown showed the opposite effects. Blocking the MEK/ERK pathway using the MEK inhibitor U0126 prevented cisplatin-induced autophagy enhanced by HK2 overexpression. Furthermore, HK2 knockdown sensitized resistance ovarian tumor xenografts to cisplatin *in vivo*. In conclusion, our data supported that HK2 promotes cisplatin resistance in ovarian cancer by enhancing drug-induced, ERK-mediated autophagy. Therefore, targeting HK2 may be a new therapeutic strategy to combat chemoresistance in ovarian cancer.

1. Introduction

Ovarian cancer, the most lethal gynecologic malignancy, ranks the fifth-leading cause of cancer death in women (Siegel et al., 2016). The current standard treatment consists of cytoreductive surgery followed by platinum/taxane-based chemotherapy (Karam et al., 2017). While most patients respond to primary chemotherapy, over 75% ultimately relapse, and subsequent chemotherapy, usually platinum-based, often fails to control the disease because of progressive development of drug resistance (Luvero et al., 2014; Ozols et al., 2003). Many mechanisms have been proposed to contribute to cancer drug resistance, including decreased drug accumulation (Kigawa et al., 1998), increased DNA repair (Zhang et al., 2009), and altered apoptotic/survival signaling pathways (Huang et al., 2011; Reles et al., 2001). Recent studies have revealed that the cytoprotective function of autophagy contributes to drug resistance in ovarian cancer (Bao et al., 2015; Spowart et al., 2012), and autophagy inhibitors can sensitize resistant cells to chemotherapy (He et al., 2015; Liang et al., 2016).

Autophagy is a natural, destructive mechanism by which cells degrade and recycle unnecessary or dysfunctional components (Mizushima and Komatsu, 2011). Under conditions of stress and nutrition deprivation, autophagy is often activated to support metabolic homeostasis and cell survival. Autophagy is considered to play a context-dependent, dual role in oncogenesis. It appears to inhibit tumor initiation by preventing oncogenic transformation. Conversely, in established tumors, it can be used as a survival mechanism to prolong cancer cell survival in unfavorable tumor microenvironments (Zhi and Zhong, 2015). Autophagy is also considered to play a crucial role in cancer cell resistance to radiation and chemotherapy (Sui et al., 2013). Cisplatin has been shown to activate cytoprotective autophagy through

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ERK in ovarian cancer cells that contributes to acquired cisplatin resistance (Bao et al., 2015; Wang and Wu, 2014); however, the molecular mechanisms involved are not fully understood.

Hexokinases phosphorylate glucose to generate glucose-6-phosphate, the initial, rate-limiting step in glycolysis. Hexokinase 2 (HK2) is an insulin-inducible isoform predominantly found in insulin-sensitive tissues such as skeletal muscles and adipose tissues (Wilson, 2003). HK2 is highly expressed in several cancers and helps drive tumor growth by maintaining the high glycolysis rates of rapidly growing tumors (Mathupala et al., 1997). In particular, HK2 is overexpressed in ovarian cancer tissues, with higher expression associated with more advanced clinical stage and poorer progression-free survival (Jin et al., 2014; Suh et al., 2014). Moreover, higher HK2 expression has been found to be associated with chemoresistance in ovarian cancer (Suh et al., 2014), suggesting that HK2 is functionally involved in chemoresistance of this grievous malignancy. In the present study, we investigated the role of HK2 in chemoresistance of ovarian cancer *in vitro* and *in vivo*. We detected increased HK2 levels in platinum-resistant human ovarian cancer cells and tissues. Cisplatin downregulated HK2 expression in cisplatin-sensitive but not resistant ovarian cancer cells. HK2 overexpression enhanced cisplatin-induced, ERK-mediated cytoprotective autophagy and increased cisplatin resistance. HK2 knockdown inhibited cisplatin-induced autophagy and sensitized resistant ovarian cancer cells to cisplatin *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and reagents

The human ovarian cancer cell line A2780 and its cisplatin-resistant derivative A2780/CP70 (Behrens et al., 1987) were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). The A2780/CP70 cell line was obtained by intermittent exposure of the A2780 line to increasing cisplatin concentrations up to 70 μM. The cells were initially exposed three times to 3 nM cisplatin for 3 days each during a 3–6-week period. After that, the dose was doubled and the procedure was repeated until the drug dose went up to 70 μM. The two cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). The resistant cell line A2780/CP70 was routinely challenged with cisplatin (Sigma Aldrich, St Louis, MO, USA). The autophagy inhibitor 3-methyladenosine (3-MA) was purchased from Sigma. The MEK inhibitor U0126 was obtained from Promega (Madison, WI, USA).

2.2. Tissue samples

Tumor tissues were collected from ovarian cancer patients admitted to Obstetrics and Gynecology Hospital of Fudan University. All patients were treated with surgery followed by standard platinum-based chemotherapy. Progression-free survival (PFS) was defined as the time from surgery to progression or recurrence. Patients with a PFS < 6 months were considered platinum resistant and those with a PFS > 6 months as platinum sensitive. The study was approved by the Ethics Committee at Obstetrics and Gynecology Hospital of Fudan University.

2.3. Immunohistochemistry

Tumor tissues were fixed in formalin, embedded in paraffin, and cut into 4-μm sections. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and boiled in citrate buffer (10 mM, pH 6.0) for 10 min for antigen retrieval. The samples were subsequently treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidases. After blocking in 5% bovine serum albumin (BSA; Boster Bioengineering, Wuhan, China), the slides were incubated with an HK2 polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C for 1 h followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody for 20 min. The slides were visualized with DAB (3,3′-diaminobenzidine), counterstained with hematoxylin, and subjected to microscopic analysis.

2.4. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from cells and tissue samples using Trizol (Invitrogen). cDNA was synthesized using the PrimeScriptVR RT reagent Kit (Takara, Dalian, China). The RT-qPCR was performed using the SYBR Premix Ex TaqTM II reagent kit (Takara). The mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to β-actin. The primers used in the PCR are: HK2, 5′-ATCCTGCAACACTTAGG GCT-3′ (Forward) and 5′-CCACACCCACTGTCACTTTG-3′ (Reverse); β-actin, 5′-CCTGAAGTACCCCATCGAGC-3′ (Forward) and 5′-AGAGGCG TACAGGGATAGCA-3′ (Reverse).

2.5. Western blot analysis

The cells and tissue samples were lysed in RIPA lysis buffer (Beyotime, Jiangsu, China). The protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Lysate samples (30 μg each) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking in 5% non-fat milk in PBS for 1 h, the membranes were incubated with primary antibodies toward HK2 (1:1000; Santa Cruz Biotechnology), LC3 (1:1000; Cell Signaling Technology), p62 (1:500), *p*-ERK1/2 (1:200), and ERK1/2 (1:1000; Abcam, Cambridge, MA, USA), respectively, at 4 °C overnight. After washing in PBS, the blots were incubated with HRP-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence reagent (Pierce) and quantified by densitometric analysis using ImageJ.

2.6. Lentivirus constructs and infection

For HK2 knockdown, a short hairpin RNA (shRNA) targeting HK2 (shHK2, GGACCTTGGAGGAACAAATTT) was synthesized and cloned into the lentiviral vector pGCSIL-GFP (Genechem, Shanghai, China). The lentiviral vector carrying a scrambled shRNA served as negative control (shCtrl). For HK2 overexpression, the coding sequence of HK2 was amplified by PCR and inserted into the lentivirus vector GV208 (Genechem) to generate the Lenti-HK2 vector. The empty GV208 vector served as negative control (Lenti-EV). The lentiviral vectors were co-transfected with pHelper1 and pHelper2 vectors into 293T cells using Lipofectamine 2000. Viral supernatants were collected 48 h after transfections, cleared through a 0.45-μm filter, and ultra-centrifuged. The human ovarian cancer cells were infected with lentiviral particles at 30 multiplicity of infection (MOI) for 48 h for HK2 knockdown or overexpression.

2.7. MTT assays

Cells were seeded at a density of 5×10^3 cells per well in 96-well plates. After overnight incubation, the cells were treated with cisplatin for 48 h. To test the effects of 3-MA, cells were incubated with 3-MA (1 mM) for 30 min prior to cisplatin treatment. The cells were subsequently incubated with MTT (Sigma) for 4 h. OD at 570 nm was recorded on a Vmax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.8. Clonogenic assays

Cells were plated at a density of 1.5×10^3 cells per well in six-well dishes and allowed to adhere overnight. After treatment with 10 μM cisplatin for 24 h, the cells were cultured for 10–14 days until large

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