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Original Article

Entinostat reverses cisplatin resistance in esophageal squamous cell carcinoma via down-regulation of multidrug resistance gene 1

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ABSTRACT

Cisplatin resistance frequently occurs in esophageal squamous cell carcinoma (ESCC). The underlying mechanism for cisplatin resistance in ESCC remains largely obscure. Here we report that entinostat reversed cisplatin resistance in ESCC both in vitro and in vivo by induction of apoptosis and inhibition of cell proliferation, accompanied by a decrease of multidrug resistance gene 1 (MDR1), P-Src, Mcl-1, Cyclin D1 and an increase of cleaved PARP. MDR1 expression was associated with worsen survival of ESCC patients with cisplatin-based chemotherapy. Dasatinib potentiated entinostat to overcome cisplatin resistance. By inhibiting Src, dasatinib reduced the expression of MDR1 and Mcl-1. Furthermore, Obatoclax, an inhibitor of Mcl-1, obviously decreased the expression of MDR1, suggesting that entinostat might surmount cisplatin resistance in ESCC via a Src-Mcl-1-MDR1 pathway. Interestingly, cisplatin also enhanced the effect of entinostat both in vitro and in vivo. Our data disclose a molecular basis that entinostat reverses cisplatin resistance, and provide a promising strategy with combinatorial drugs to treat cisplatin resistant ESCC patients.

Introduction

Cisplatin resistance frequently occurs during cisplatin-based treatment [1], and might be caused by transportation of cisplatin outside of cells, in which MDR1 play a major role [2]. Many factors affect MDR1 to alter cisplatin sensitivity in cancer cells. Human helicase RECQL4 drove cisplatin resistance in gastric cancer by activating an AKT-YB1-MDR1 signaling pathway [3]. SH3GL1 inhibition reversed multidrug resistance in colorectal cancer cells by down-regulating MDR1/P-glycoprotein via EGFR/ERK/AP-1 pathway [4]. By inhibiting Src, dasatinib overcame multidrug resistance via decreased MDR1 in human multidrug resistant myeloma cells [5]. Obatoclax reversed the cross-resistance in cisplatin-resistant non-small cell lung cancer cells via inhibiting Mcl-1 [6]. However, the mechanism underlying cisplatin resistance in ESCC remains uncertain.

Targeting epigenetic routes was an effective strategy to overcome chemo-resistance [7]. Entinostat, an epigenetic drug, affects many pivotal targets and exhibits pronounced anti-cancer functions in human cancer [8–15], with little side effects on normal cells [16], and is well tolerated by patients in clinic [15,17], thus it has great potential to be used in the treatment of cancer patients. To date, no reports showed any relationship between entinostat and cisplatin resistance in ESCC. In this study, we explore if entinostat is a promising agent to overcome cisplatin resistance in ESCC.

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Materials and methods

Cell culture

Human ESCC cell lines KYSE520, KYSE180, KYSE410, KYSE30 and EC109 were obtained from the American Type Culture Collection (Rockville, MD). HKESC1, a human esophageal squamous cell carcinoma cell line, was kindly provided by Professors G. Srivastava and G.S. Tsao from University of Hong Kong [18]. Cell lines were clear of *Mycoplasma* as determined by the Lonza kit (LT-07-418) within 6 months of their use. All cells were cultured in a 37 °C incubator with humidity and 5% CO_2.

Antibodies and immunoblotting

The immunoblotting assay was performed according to routine procedures [9]. The antibodies including Anti-Acetyl-Histone H3 (Lys9) Rabbit mAb (Catalog Number, 9649), Mcl-1 Antibody (Catalog Number, 4572), Phospho-Src Family (Tyr416) (D49G4) Rabbit mAB (Catalog Number, 6943), Src antibody (Catalog Number, 2108), PARP antibody (Catalog Number, 9542), Cyclin D1 antibody (Catalog Number, 2922S), Phospho-Histone H2A.X (Ser 139) (20E3) Rabbit mAb (Catalog Number, 9718S), Histone H3 (D1H2) Rabbit mAb (Catalog Number, 4499S) and MDR1/ABCB1 (D3H1Q) Rabbit mAb (Catalog Number, 12683S) were purchased from Cell Signaling Technology (Beverly, Massachusetts). Monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Catalog Number, KC-5G4) was purchased from Kanchen Incorporation (Shanghai, China), Monoclonal anti-beta-actin mouse antibody (Catalog Number, A3853) was purchased from Sigma (St. Louis, USA).

Cell proliferation assay

The CellTiter96 AQ Non-Radioactive Cell Proliferation Kit (Promega, Madison, Wisconsin) was used to determine cell viability, following the manufacturer's instructions and routine procedure [9]. Briefly, $1–2 \times 10^4$ cells were plated onto 96-well plates for 24 h. Cells were then grown in either control medium with equivalent solvent DMSO or the same medium containing respected reagents, and incubated for another 72 h. After reading at 490 nm with a microplate reader, the percentages of surviving cells from each group relative to controls, defined as 100%, were determined by reduction of MTS.

Quantification of apoptosis

An ELISA apoptosis kit (Promega, Madison, Wisconsin) was used to quantitatively measure cytoplasmic histone associated DNA fragments, showing the level of apoptosis, following the manufacturer's guidelines.

Tumor graft models and in vivo treatments

The experimental protocol was approved by Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer Center. Male BALB/c mice (4–6 weeks old) were implanted with EC109 cells (5×10^6 cells in PBS) into the foreleg of each mouse. After the xenograft has formed with the volume of approximately 200 mm^3, the animals were injected with cisplatin intraperitoneally twice a week, with incremental dosage from 1 to 2.5 mg/kg in approximately 15 days, to form cisplatin resistance in vivo. Then, the animals were divided into four groups as follows: drug free control group (6 animals); entinostat group (5 animals); cisplatin group (4 animals); entinostat+cisplatin group (4 animals). Cisplatin (2.5 mg/kg) was injected intraperitoneally, and entinostat (5 mg/kg) was administered by oral gavage twice a week. The volumes of xenografts were measured ($(a \times b^2)/2$)once every two days for 11 days (Note: a, length of the tumor; b, width of the tumor). At the end of the experiments, the animals were killed, the xenografts were extracted, the weights were measured, and the extracted xenografts were photographed. HE staining was used to show the pathological status of the xenografts. Immunoblotting assay was performed to examine the expression of interest proteins in the xenografts.

Patients, specimens and significance of MDR1

The ESCC specimens from 28 patients with cisplatin-based chemotherapy were obtained from Department of Specimen Storage, Sun Yat-Sen University Cancer Center. The samples were randomly assigned to experimental groups and the investigators were blinded to the same group allocation during the experiment. The clinical data and follow-up history were retrieved from hospital databases. The protocol was approved by Ethics Committee of Sun Yat-Sen University Cancer Center, and written informed consent was obtained from each patient. Immunoblotting assay was performed to examine protein expression in the samples.

Statistical analyses

Statistical analyses were performed using a two-sided Student's *t* test when appropriate. The association between MDR1 expression and different clinicopathologic characteristics of the ESCC patients were evaluated by Fisher's exact test as appropriate. The Kaplan-Meier method was used for estimating probability of survival and for univariate analysis. The log-rank test was used to assess the significance of difference between pairs of survival probabilities. The Cox proportional hazard model was used to evaluate the association between MDR1 and patients' survival. Data represent mean ± SEM. A p-value <0.05 is considered statistically significant. All the experiments repeated at least three times.

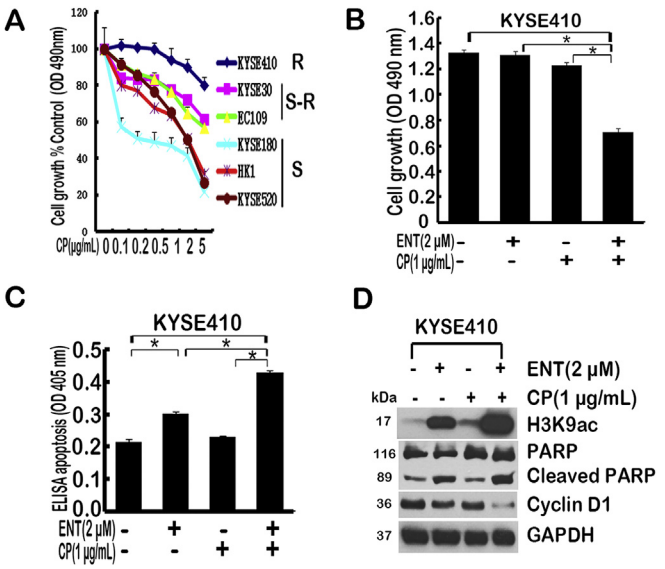

Fig. 1. Entinostat reverses cisplatin resistance in ESCC. A. MTS assay to show cisplatin sensitivity of ESCC cells. B and C. MTS and ELISA apoptotic assay exhibit entinostat to reverse cisplatin resistance. D. Immunoblotting assay to reveal the expression of candidate molecules. Note: CP, cisplatin; ENT, entinostat; R, resistant to cisplatin; S-R, semi-resistant to cisplatin; S, sensitive to cisplatin; *, P < 0.05.