Pathology - Research and Practice 214 (2018) 1467–1473

Contents lists available at ScienceDirect

Pathology - Research and Practice

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

Correlation between adenosine triphosphate (ATP)-binding cassette transporter G2 (ABCG2) and drug resistance of esophageal cancer and reversal of drug resistance by artesunate

Lei Wang[a], Liang Liu[b,*], Yuetong Chen[b], Yu Du[c], Jing Wang[b], Jianghui Liu[b]

[a] Department of Thoracic Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, 050011, PR China
[b] Tumor Institute, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, 050011, PR China
[c] Department of CT, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, 050011, PR China

ARTICLE INFO

Keywords:
Esophageal cancer
Artesunate
Multidrug resistance
Adenosine triphosphate (ATP)-binding cassette transporter G2 (ABCG2)
Animal experiment

ABSTRACT

The present study investigated the correlation between the abnormal expression of adenosine triphosphate (ATP)-binding cassette transporter G2 (ABCG2) in esophageal cancer and the drug resistance of esophageal cancer, the reversal effect in drug resistance of artesunate (Art), and the mechanism underlying esophageal cancer using nude mice with subcutaneous xenograft as an animal model. The gene and protein expression of ABCG2 was detected in 80 cases of esophageal cancer, atypical dysplasia, and normal mucosa. A subcutaneous xenograft tumor mouse model was established by subcutaneous inoculation of esophageal cancer cell line Eca109/ABCG2 into BALB/c nu/nu nude mice. The reversal of drug resistance by Art in esophageal cancer was studied *in vivo*. The mice model was injected intraperitoneally with Art and/or doxorubicin (ADM). The animals were divided into six groups: high dose Art (50 mg/kg), low dose Art (25 mg/kg), ADM (1 mg/kg), ADM and high-dose Art, ADM and low-dose Art, and physiological saline as a control. ABCG2 protein expression and cellular ADM concentration were detected by flow cytometry. The mRNA and protein expressions of ABCG2 in esophageal cancer were significantly higher than that in the normal esophagus ($P < 0.01$). The volume and mass of the subcutaneously implanted tumors in the ADM + Art high- and low-dose groups were significantly decreased than that in the control group ($P < 0.05$), while the apoptosis rate of tumor cells and the cellular concentration of ADM were increased significantly ($P < 0.05$), and the ABCG2 protein expression in the tumor cells decreased significantly ($P < 0.05$). Abnormally high expression of ABCG2 in esophageal cancer tissues is involved in the multidrug resistance of esophageal cancer. *In vivo* studies showed that Art could reverse the esophageal cancer drug resistance by regulating the ABCG2 expression in tumor cells.

1. Introduction

Chemotherapyis usually accompanied by multidrug resistance (MDR), which jeopardizes its efficacy and prognosis. Previous studies demonstrated that the MDR of tumors is correlated to a cell transmembrane protein, a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily. ABCG2 is one of the ABC superfamily members involved in MDR that has been studied in recent years. Although several studies have reported that ABCG2 is associated with MDR in a variety of tumor cells [1–3], only a few studies have suggested a correlation between ABCG2 and MDR in esophageal cancer. ABCG2 is also considered as a marker of cancer stem cells [4,5]. In the present study, we investigated the correlation between ABCG2 and MDR in esophageal cancer and conducted a preliminary search for agents that reverse the MDR in esophageal cancer.

The MDR reversal agents of tumor have been studied extensively, and a large number of these agents have been developed against the ABC family. However, due to the severe side effects, these agents have not been used widely in clinical practice [6,7]. Artesunate (Art) is an anti-malarial drug in China with satisfactory efficacy in patients with severe and drug-resistant malaria [8–10]. In addition to the anti-malarial effect, some studies also demonstrated the anti-tumor effect of Art

Abbreviations: ABCG2, adenosine triphosphate-binding cassette transporter G2; ADM, art and/or doxorubicin; AML, acute myeloid leukemia; Art, artesunate; ATP, adenosine triphosphate; EB, ethidium bromide; FBS, fetal bovine serum; FCM, flow cytometry; IHC, immunohistochemistry; MDR, multidrug resistance; RT-PCR, reverse transcription polymerase chain reaction; SCC, squamous cell carcinoma

* Corresponding author.
E-mail address: aliangdaziran@163.com (L. Liu).

https://doi.org/10.1016/j.prp.2018.08.001
Received 9 April 2018; Received in revised form 23 July 2018; Accepted 6 August 2018

[2,11]. Previous studies in our laboratory showed that Art inhibits the growth of esophageal cancer cells [12], indicating an anti-esophageal cancer effect. Also, we demonstrated the drug resistance reversal effect of Art on tumor cells [13]. The present study primarily investigated whether Art can reverse the MDR of esophageal cancer induced by ABCG2 and the underlying mechanisms. Thus, an animal model was established by inoculating the drug-resistant esophageal cancer cell line Eca109/ABCG2 into the nude mice subcutaneously. This drug-resistant esophageal cancer model was utilized for *in vivo* studies about the drug-resistant reversal effect of Art on esophageal cancer and the underlying mechanism.

Due to low toxicity and high efficiency, the development of Art into a tumor MDR reversal agent would be a promising and rewarding endeavor with a wide range of applications.

2. Materials and methods

2.1. Clinical sample preparation

A total of 150 clinical cases of esophageal squamous cell carcinoma (SCC) were collected from the Fourth Hospital of Hebei Medical University from January 2008 to November 2009. Each clinical case included esophageal squamous carcinoma tissues, adjacent tissues, and normal tissues. Fresh esophageal tissues were obtained during surgery and divided into three groups: one was stored in liquid nitrogen for reverse transcription-polymerase chain reaction (RT-PCR) detection, and the other two were fixed with 4% paraformaldehyde solution for immunohistochemistry (IHC) and flow cytometry (FCM) detection. All the selected cases present complete clinical and pathological data and have been diagnosed with esophageal SCC. Among the selected cases, 80 consisted of esophageal SCC tissues, 80 atypical dysplasia tissues, and 80 normal esophageal mucosa tissues (55 male and 25 female cases, average age 58.38 ± 6.70 years and median age 58.5 years). Among the 80 cases of atypical dysplasia tissues, 19 were grade I, 36 grade II, and 25 grade III. The 80 cases of esophageal SCC tissues were characterized as follows: 59 cases were highly differentiated and 21 poorly differentiated, 54 exhibited infiltration into the fibrous membrane and 26 had not reached the fibrous membrane, and 27 cases were associated with lymph node metastasis and 53 not associated with metastasis. The present study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) (No. 2016MEc016). Informed consent was obtained from all individual participants included in the study.

2.2. Experimental animals

Male and female (1:1) BALB/c nu/nu nude 4-week-old mice, weighing 17–20 g, were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences [Animal ID: SCXK 2005-0013] and maintained in a clean animal room [specific pathogen free (SPF) grade] of the Animal Laboratory Center of the Fourth Hospital of Hebei Medical University. The room temperature and relative humidity were maintained at 25 ± 1 °C and 40–60%. Animal experiments were conducted according to the Institutional Animal Care and Use Committee guidelines.

2.3. Detection of the mRNA expression levels of ABCG2 in different esophageal lesions by RT-PCR

Total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). The reverse transcription kit (A3500, Promega, USA) was used for cDNA synthesis that was utilized as a template for PCR amplification by Taq DNA polymerase (Promega,USA). The primer sequences were as follows: *ABCG2*: upstream primer 5′-GGTCAGAGT GTGGTTTCTGTAGCA-3′, downstream primer 5′-GTGAGAGATCGATG CCCTGCTTTA-3′; *GAPDH*: upstream primer 5′-ACCACAGTCCATGCCA TCAC-3′, downstream primer 5′-TCCACCACCCTGTTGCTGTA-3′. A volume of 8 μL PCR product was subjected to 1.5% agarose gel electrophoresis, ethidium bromide (EB) (Sigma-Aldrich, St. Louis, MO, USA) staining, and imaging by the UV gel imaging system. The absorbance (A) value of the PCR product was analyzed by Gel-pro Analyzer 3.1 software, and the ratio between the A value of *ABCG2* and that of *GAPDH* was calculated to analyze the expression level of *ABCG2* mRNA semiquantitatively.

2.4. Detection of the protein expression of ABCG2 in different esophageal lesion tissues by FCM

A single cell suspension was prepared using a mesh rubbing method, and the cell concentration was adjusted to 1×10^7/mL. Subsequently, 0.1 mL single cell suspension was mixed with 0.01 mL of FITC-labeled ABCG2 antibody [Biolegend, USA] for 30 min at room temperature, followed by detection using flow cytometry (FC500, Beckman-Coulter, USA).

2.5. Detection of the protein expression of ABCG2 in different esophageal lesion tissues by IHC

The experiment was conducted using the conventional SP method. Briefly, the paraffin-embedded tissues were sliced into 4-μm-thick sections and incubated with mouse anti-human ABCG2 monoclonal antibody [ABCG2 (5D3) antibody (sc-18841) Santa Cruz Biosciences, USA] (diluted 1:100), and then, with the biotinylated goat anti-mouse IgG. The cells with brown-yellow granules in the cell membrane or cytoplasm were considered as ABCG2-positive cells. A total of 10 fields were selected randomly, and 100 cells were selected for recording the number of ABCG2-positive cells. Consequently, the percentage of ABCG2-positive cells, as well as the average percentage among the 10 fields was estimated. A comprehensive evaluation was carried out according to the intensity of tumor cell staining and the percentage of the positive cells. The number of positive cells > 10% is considered as positive, and the absence of positive cells or that ≤10% was considered as negative.

2.6. Preparation of drug-resistant esophageal cancer cell line Eca109/ABCG2 and establishment of nude mice model with subcutaneous xenograft

Human esophageal cancer drug-resistant Eca109/ABCG2 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Paisley, Auckland) and 300 mg/L G418 (Gibco-BRL, Life Technologies, Paisley, Auckland) in a 37 °C incubator supplied with 5% CO_2. G418 was removed from the culture medium 1 week before the experiment. Subsequently, the cells in the logarithmic growth phase were adjusted to a density of 3×10^7 cells/mL and inoculated subcutaneously (200 μL/animal) in the left forelimb of nude mice.

2.7. Animal experiment grouping and drug intervention

A total of 36 nude mice were randomly and equally divided into six groups according to the body weight: half males and half females. Nude mice were injected subcutaneously for 1 week with the cancer cells mentioned above, and the tumor formation rate was 100%. The ADM group was administered 1 mg/kg ADM; the Art high- and low-dose groups received 50 mg/kg and 25 mg/kg Art, respectively; the ADM + Art low-dose group was administered 1 mg/kg ADM and 25 mg/kg Art, respectively; the ADM + high-dose group was administered 1 mg/kg ADM and 50 mg/kg Art, respectively; the control group was subjected to an equivalent volume of saline. Art was intraperitoneally injected one time daily for 7 days consecutively, followed by no drug for 1 week, and then resuming the regimen for 7 days. ADM was injected intraperitoneally one time every 3 days for 7 cycles.