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Tetrandrine enhances cytotoxicity of cisplatin in human drug-resistant esophageal squamous carcinoma cells by inhibition of multidrug resistance-associated protein 1

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Abstract. Multidrug resistance is one of the major causes limiting the efficacy of chemotherapeutic agents to control esophageal cancer. Herein, we investigated that the effect and mechanism of tetrandrine (TET) in the human esophageal squamous carcinoma cisplatin-resistant cell line YES-2/DDP. The human esophageal squamous carcinoma cisplatin-resistant cell line YES-2/DDP was isolated by stepwise selection in increasing concentrations of cisplatin. The CCK-8 method was carried out to measure the cell viability when cells were exposed to TET with or without cisplatin, and the IC_{50} and resistance index (RI) of cisplatin was then calculated. Real-time RT-PCR and western blotting were used to detect the mRNA and protein expression of multidrug resistance 1 (MDR1), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP), respectively. Flow cytometry was adopted to determine CMFDA efflux and cell apoptosis, respectively. The resulting cell line YES-2/DDP was 16.4-fold resistant to cisplatin, the cytotoxicity of cisplatin to YES-2/DDP cells was enhanced by TET in a dose-dependent manner. Further, it was found that the expression of MDR1 and BCRP was similar in different treated cells. In contrast, the expression of MRP1 was markedly increased in YES-2/DDP cells, which was dose-dependently decreased by TET. In agreement with the results, MRP1 activity was also reversed by TET. In conclusion, TET possesses a reversal effect on drug resistance in YES-2/DDP cells through down-regulation of MRP1, and has the potential to be an adjunct to chemotherapy for esophageal cancer.

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Key words: tetrandrine, multidrug-resistant, oesophageal squamous carcinoma, multidrug resistance-associated protein 1

Introduction

Chemotherapy is regarded as an important line of defense against esophageal cancer which is one of the most aggressive and lethal malignancies. However, on account of drug resistance especially multi-drug resistance (MDR), only a limited proportion of cancer patients respond favorably to commonly used chemotherapeutic drugs (1). With respect to the mechanisms of drug resistance, ATP-binding cassette (ABC) transporters, such as ABCB1/multidrug resistance 1 (MDR1), ABCC1/multidrug resistance-associated protein 1 (MRP1) and ABCG2/breast cancer resistance protein (BCRP), mediate energy-dependent drug efflux and play a main role in chemoresistance (2,3). Therefore, it seems imperative to find new drugs or methods especially targeting ABC transporters to reverse tumor drug-resistance.

Tetrandrine (TET) (Fig. 1), a bis-benzylisoquinoline alkaloid isolated from the Chinese herb 'Han-Fang-Ji' (Radix *Stephania tetrandra* S. Moore), has been found to have immunosuppressive, free radical scavenging and anti-inflammatory activities (4-6). Furthermore, many recent studies have shown that TET exerts antitumor effects (7,8). In addition to inhibiting proliferation and inducing apoptosis of several cancer types, TET has exhibited potential as an adjunct to chemotherapy in many drug-resistant cancer cell lines (9,10). However, it remains unclear whether TET can reverse ABC transporter-mediated drug efflux. Moreover, it is also unknown whether TET can be used as an adjunct to chemotherapy for esophageal cancer.

In this study, a human esophageal squamous carcinoma cell line (YES-2) was selected by stepwise exposure to increasing concentrations of cisplatin to produce a cisplatin-resistance esophageal cancer cell line (YES-2/DDP). We examined whether TET can effectively reverse cisplatin resistance in YES-2/DDP and evaluated its possible mechanisms.

Materials and methods

Materials. TET ($C_{38}H_{42}O_6N_2$, MW:622.8, purity ≥98%) determined by HPLC as previously described (11) was the product of Sigma Chemical Company (St. Louis, MO, USA). Cisplatin

was obtained from Qilu Pharmaceutical Company (Shandong, China). MDR1, MRP1 and BCRP were purchased from Chemicon International (Temecula, CA, USA), and β-actin antibodies were products of Cell Signaling Technology (Boston, MA, USA). Bicinchoninic acid (BCA) protein assay kit and enhancer chemiluminescent (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL). Cell Counting Kit-8 (CCK-8) and Annexin V-FITC Apoptosis detection kit were purchased from Beyotime Institute of Biotechnology (Nanjing, China) and KeyGen Biotech (Nanjing, China) respectively. Chloromethylfluorescein diacetate (CMFDA) were obtained from Invitrogen (Carlsbad, CA, USA).

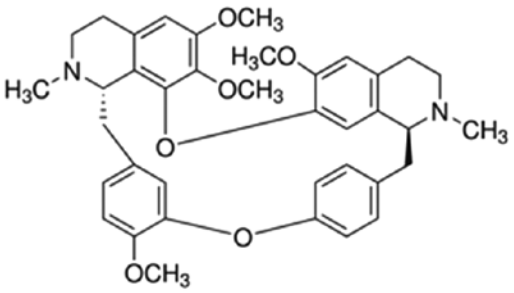

Figure 1. Chemical structure of TET.

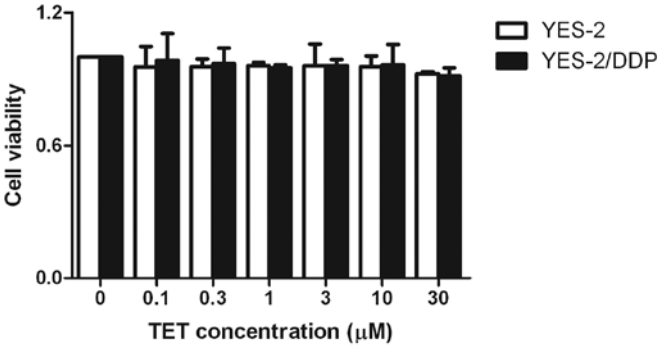

Figure 2. Effect of TET on cell viability of YES-2 or YES-2/DDP cells. Cells were treated with an increasing concentration of TET for 72 h; the cell viability was measured by CCK8.

Cell lines and cell culture. YES-2 cells, a human esophageal squamous carcinoma cell line, and its cisplatin-resistant cell subline, YES-2/DDP, were cultured in DMEM medium with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml), and maintained at 37˚C in a humidified atmosphere of 5% CO_2. The cisplatin-resistant cells of YES-2/DDP were isolated by stepwise selection in increasing concentrations of cisplatin starting with 0.01 μg/ml. When cells became confluent in medium containing cisplatin, the drug concentration was increased to 0.03, 0.05, 0.1, 0.3, 0.5, and 1 μg/ml, the maximal concentration used. The YES-2/DDP cell subline was passaged in cisplatin-free medium and remained stably resistant to cisplatin for several months. To prevent the outgrowth of revertants, the cells were periodically reselected in the presence of 0.1 μg/ml cisplatin. Under these conditions no change in resistance was observed over 1.5 years.

Cell viability. The effects of TET and/or cisplatin on the growth of YES-2/DDP and YES-2 cells were measured by CCK-8 method. The cells were dispensed in 96-well plate at a density of $1x10^5$ cells per well. After 24 h of incubation, they were treated with different concentration of TET and/or cisplatin, and were cultured for 72 h. After such treatments, the cells were incubated with 20 μl CCK-8 for 2 h at 37˚C, and then measured the absorbance at 450 nm using model 550 microplate reader (Bio-Rad, USA). The cell growth inhibition was determined by triplicate assays. The half of inhibition concentrations (IC_{50}) values were calculated from cytotoxicity curves. The resistance index (RI) was calculated by dividing the IC_{50} for MDR cells by that for parental sensitive cells.

Apoptosis of cells. To measure the apoptosis of cells, annexin V-FITC Apoptosis detection kit was used. Annexin V binding on the surface of apoptotic cells expressing phosphatidylserine and propidium iodide (PI) incorporation by dead cells were analyzed by using standard protocols. Briefly, cells were detached by trypsin treatment, resuspended in PBS at a concentration of 10^5 cells/ml, and labeled with 5 μl Annexin V-FITC for 10 min. After addition of 10 μl PI, the samples were analyzed by flow cytometry.

Quantitative reverse transcription-PCR. Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized. The reverse transcription was conducted by incubating for 60 min at 43˚C followed by 10 min at 70˚C. SYBR® Premix Ex Taq™ (Takara) was used to quantitatively monitor the accumulation of DNA products. Melting curves were performed to assure that the fluorescence was derived from dye intercalating into a specific, homogeneous amplification product. For amplification primers of MDR1, MRP1 and BCRP were 5'-GTG TTT CTG GTC AGC CCA ACT-3' (sense) 5'-TTG GAT CTC AGG ATG GCT AGG-3' (antisense); 5'-GTG TTT CTG GTC AGC CCA ACT-3' (sense) and 5'-TTG GAT CTC AGG ATG GCT AGG-3' (antisense); 5'-ACG AAC GGA TTA ACA GGG TCA-3' (sense) and 5'-CTC CAG ACA CAC CAC GGA T-3' (antisense), respectively. The primers used for amplification of GAPDH cDNA as an internal standard were 5'-CCA CCC ATG GCA AAT TCC-3' (sense) and 5'-TGG GAT TTC CAT TGA TGA CAA-3' (antisense). Each reaction contained 12.5 μl of SYBR Premix Ex Taq, 0.5 μl of each primer, and 2 μl of template made up to 20 μl with filter sterilized water. Real-time PCR was performed on a CFX96 Real-time detection systems (Bio-Rad, USA) with initial denaturation at 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, 72˚C for 30 sec and 85˚C fluorescent signal acquirement. Relative expressions were determined via the *Ct* method normalized to MDR1, MRP1, BCRP or GAPDH standards.

Western blotting. The different treated cells were harvested. Total proteins were prepared according to the method described by the protein extract kit (Piece Biotechnology, Rockford, USA). Protein concentrations were determined by BCA protein assay kit. Protein extracts were fractionated on 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5%

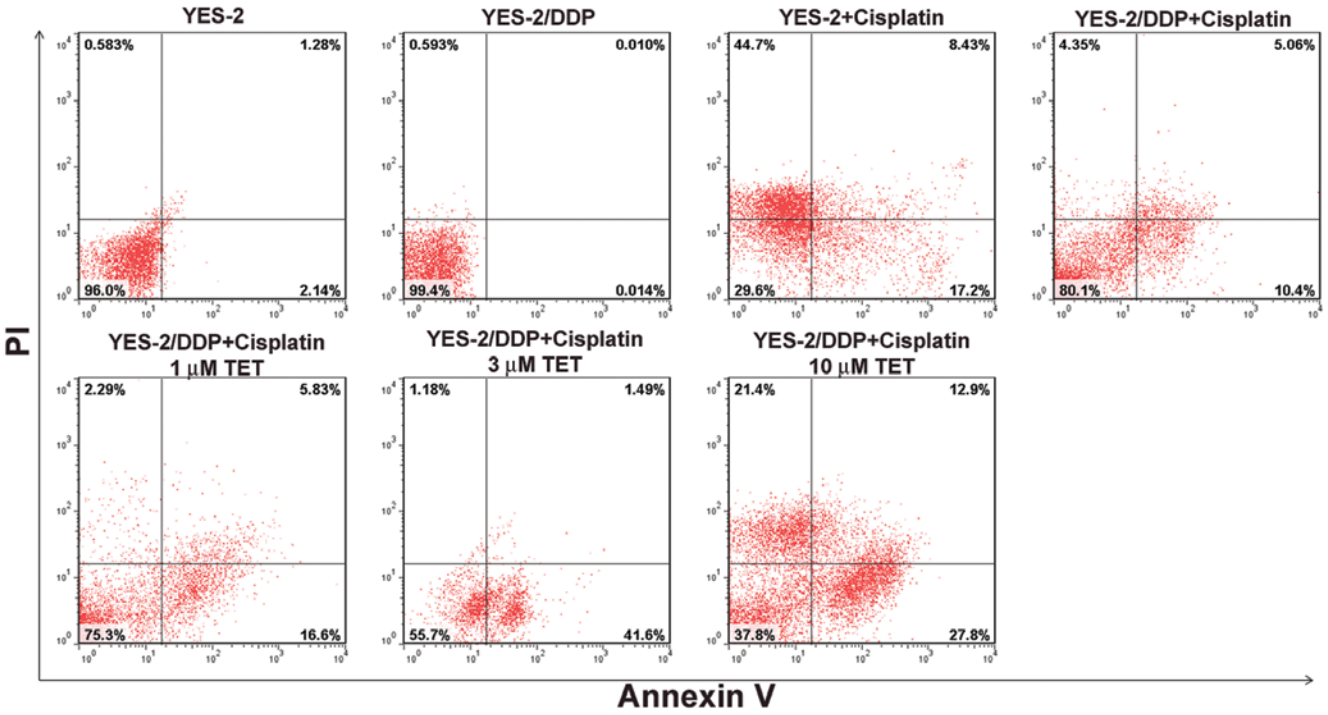

Figure 3. Effect of TET on the cisplatin-induced apoptosis of YES-2/DDP cells. Cells were treated with cisplatin (10 μg/ml) and TET (1, 3, 10 μM, respectively) for 72 h; the apoptosis of cell was measured with a PI and Annexin V kit by flow cytometry.

(w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by incubation with a rabbit primary polyclonal antibody at 4˚C overnight. Then the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000). Antibody binding was visualized with an ECL chemiluminescence system and short exposure of the membrane to X-ray film (Kodak, Japan).

MRP1 activity assay. To assess MRP1 activity, a MRP specific probe CMFDA(12) was used to detect intracellular CMFDA accumulation, which is similar to functional activity of drug efflux pumps. Single cell suspensions obtained by trypsinization from confluent monolayers of YES-2/DDP or YES-2 cells, were incubated at 37˚C for 60 min in the presence of TET and cisplatin in serum-free DMEM containing CMFDA 1.0 μmol/l, then washed three times with ice-cold PBS, resuspended in ice-cold PBS containing 1% fetal bovine serum and kept on ice until the analysis by flow cytometry.

Statistical analysis. Results were analyzed using Student's test or by ANOVA where appropriate. All data in this study were expressed as mean ± SD. P-values ≤0.05 was considered significant.

Results

The effect of TET on cell viability of YES-2 or YES-2/DDP cells. We first examined the cell viability and cytotoxicity of TET itself on the cisplatin-resistant cell line YES-2/DDP and its parental cell line YES-2. Both cell lines were pretreated with increasing concentration of TET from 0.1 to 30 μM for 72 h, respectively. There were no obvious cytotoxicity on YES-2/DDP and YES-2 treated by varying dose of TET (Fig. 2). Cell survival was >95% in both YES-2 and YES-2/DDP cells when exposed to 10 μM or lower concentrations of TET. Therefore, TET concentrations of 1, 3, and 10 μM were used in following experiment.

Table I. Effect of TET on the IC_{50} and RI of cisplatin in YES-2/DDP cells.

Cell treatment	IC_{50} (μg/ml)	RI
YES-2	0.97	1.0
YES-2/DDP	15.91	16.4
YES-2/DDP + 1 μg/ml TET	11.43	11.8
YES-2/DDP + 3 μg/ml TET	8.62	8.9
YES-2/DDP + 10 μg/ml TET	4.57	47.0

The reversal effect of TET on resistance to cisplatin in YES-2/DDP cells. In order to investigate whether TET modulated the sensitivity of cells to cisplatin, YES-2 or YES-2/DDP cells were incubated with various concentrations of TET and a full range of concentrations of cisplatin for 48 h. The IC_{50} and RI of cisplatin in the different treated cells were evaluated by CCK-8. As demonstrated in Table I, it was clear that the sensitivity to cisplatin in YES-2 cells was significantly more than that in YES-2/DDP cells, and TET could effectively reverse the drug resistance dose-dependently. Similar outcome was also shown in the analysis of apoptosis measured by flow cytometry (Fig. 3), TET dose-dependently enhanced cell apoptosis induced by cisplatin.

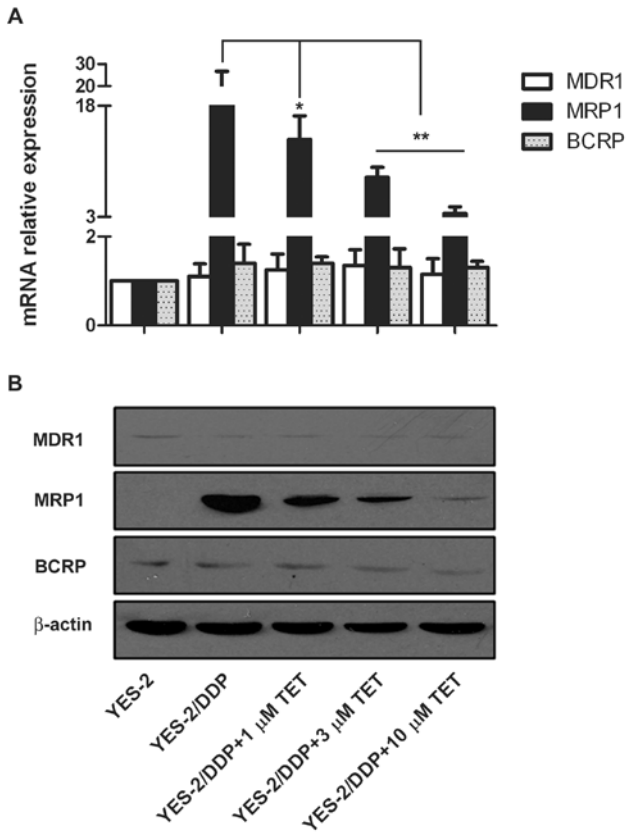

Figure 4. Effect of TET on ABC transporters expression of YES-2/DDP cells. Cells were treated with PBS or different doses of TET (1, 3, 10 μM, respectively) for 72 h; the expression of ABC transporters were determined by QRT-PCR (A) and western blotting (B). *P<0.05, **P<0.01.

The effect of TET on the expression of MDR1, MRP1 and BCRP. Since the overexpression of ABC transporters in cancers is considered to be a primary determinant of the MDR phenotype, we detected the expressions of three main ABC transporters by qRT-PCR and western blotting to find whether TET reversed the drug resistance to cisplatin through mediating these ABC transporters. It was indicated that MDR1 and BCRP expression have not obvious changes in different treated cells. In contrast, the expression of MRP1 was markedly increased in YES-2/DDP cells, which was dose-dependently decreased by TET (Fig. 4).

The effect of TET on the accumulation of intracellular CMFDA. Based on MRP1 hyperexpression on YES-2/DDP cells, we used a specific MRP1 probe CMFDA to incubate the different treated cells for 60 min. Further, we analyzed the accumulation of intracellular CMFDA in the different treated cells by flow cytometry. As shown in Fig. 5, compared with that in YES-2/DDP cells, the accumulation of intracellular CMFDA was more intense in drug-sensitive YES-2 cells. It was also demonstrated that TET exhibited dose-dependent enhancement in the accumulation of intracellular CMFDA.

Discussion

In this study, we first established a human esophageal squamous carcinoma cisplatin-resistant cell subline, YES-2/DDP, which express a high level of MRP1 mRNA and protein, and was 16.4-fold resistant to cisplatin as compared to its parent cell line, YES-2. We found that TET could effectively reverse the resistance of cisplatin in YES-2/DDP cells, and dose-dependently inhibited the expression of MRP1 but not MDR1 and BCRP. Thereby, we deduced that MRP1, 190 kDa glycoprotein mainly localized in cell membrane in almost all tissues, is considered as an ATP-dependent efflux pump and has 14% structural homology with MDR1, but differs substantially from MDR1. Its main physiological functions involve cellular transportation and secretion, protecting the body from biological damage (13,14). The overexpression of MRP1 has been reported in a variety of human malignancies, which causes increased efflux of chemotherapeutic drugs leading to the occurrence of drug resistance (15-17).

MRP1 can actively transport the drugs into subcellular organelles, or indirectly affect the distribution of drugs to reduce drug concentration in the nucleus, thereby cut down the DNA injury. MRP1 can also reduce the pH value in the cytoplasm or organelles through forming chloride ion channel or changing channel activity, which will result in the acidic environment where the protonated drugs are largely discharged. In addition, MRP1 can even shift drugs out of cells into the extracellular fluid by vesicle transportation or exocytosis (14,18,19). Numerous studies have shown that inhibition of MRP1 expression by a variety of methods eased the development of drug resistance, thus supported clinical chemotherapy (20-22). Regarding the expression of MRP1 in esophageal cancer, it has been demonstrated that MRP1 often overexpressed in different esophageal cancer cell lines or cancer tissues of patients (23-25). It was even reported that the proportion of MRP1-positive samples in the esophageal cancer was significantly higher than that in the adenocarcinomas of the stomach and the colorectal adenocarcinomas, showing MRP may play a great role in the drug resistance in esophageal cancer (26). Therefore, to find and develop drugs targeting MRP seems to be very favorable for clinical chemotherapy for esophageal cancer.

Many studies have indicated that TET possess a reversal effect on MDR in many cancer cell lines, and might enhance the efficacy of chemotherapy such as cyclosporine A, daunorubicin, etoposide, cytarabine and droloxifene (27,28). It was mostly demonstrated that TET exerted its reversal effect through downregulating MDR1. For example, in human oral cancer MDR KBv200 cells, TET enhanced the antitumor effect of vincristine via directly binding to MDR1 and increasing intracellular VCR accumulation (29). It was even reported that TET exhibited stronger activity to reverse drug resistance to daunorubicin, vinblastine and doxorubicinin in human T lymphoblastoid leukemia MDR MOLT-4 cells, when compared to well-known MDR1 inhibitor cyclosporine A (CsA) (30).

The derivatives of TET such as H1, bromotetrandrine and isotetrandrine were also found to be candidates of effective MDR reversing agent in cancer chemotherapy (31-33). In addition to the inhibitory effect of TET on MDR1 expression, it was also shown that TET could significantly inhibit MDR of tumor cells induced by chemotherapy via other means including reducing lung resistance-related protein (LRP) expression, diminishing the activity of topoisomerase II or suppress activation of NF-κB (34,35). In this study, we reported that TET had a reversal effect on drug resistance

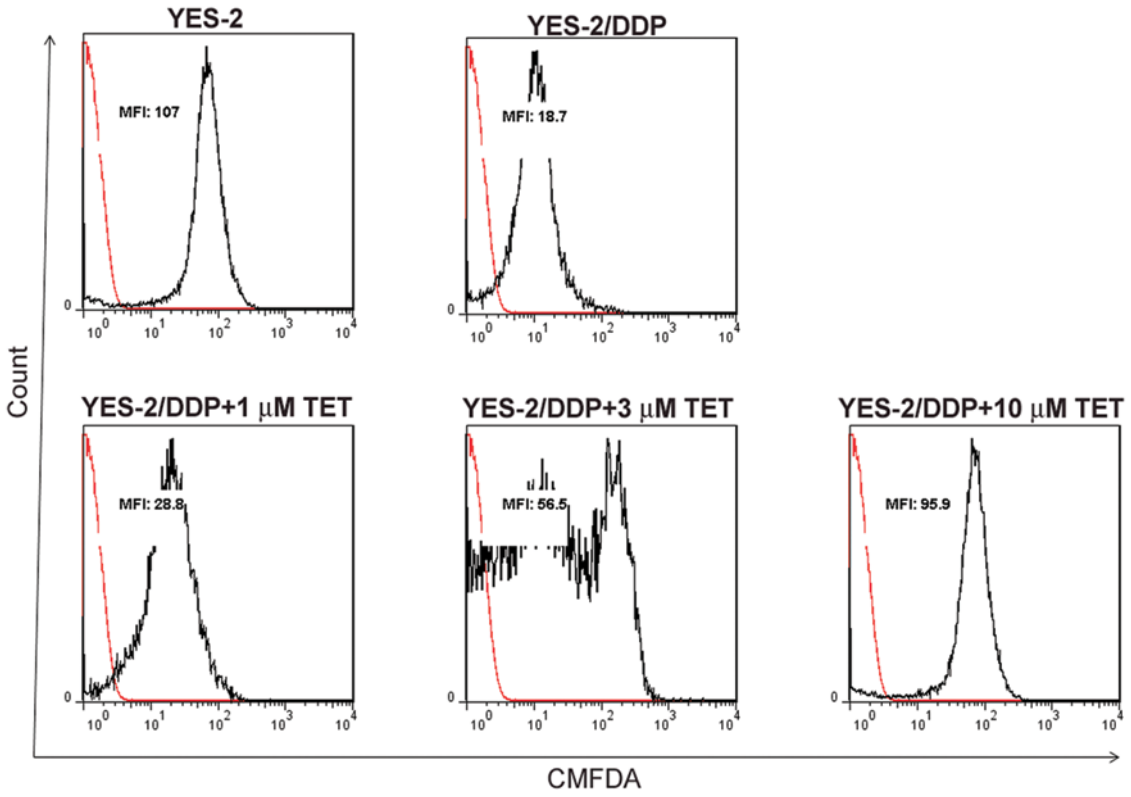

Figure 5. Effect of TET on the accumulation of intracellular CMFDA of YES-2/DDP cells. Cells were treated with PBS or different doses of TET (1, 3, 10 μM, respectively) for 72 h; then cells were loaded with CMFDA for 1 h, cells were washed with PBS three times. Cells were further incubated CMFDA-free medium for 30 min, the mean fluorescence intensity (MFI) of CMFDA were assayed by flow cytometry and indicated as the accumulation of intracellular CMFDA.

to cisplatin in MDR human esophageal cancer YES-2/DDP cells. Compared with untreated YES-2/DDP cells, TET-treated YES-2/DDP cells got more sensitivity to cisplatin. Moreover, further study suggested that TET probably intervened in occurrence of drug resistance by inhibiting MRP1 expression and activity, indicating TET might be considered as a potential adjunct to chemotherapy for esophageal cancer overexpressing MRP1. However, regardless whether TET targets MDR1 or MRP1, the detailed molecular mechanism which TET modulates MDR needs to be further investigated, and has now been thought as the key unsolved question which influences further development of TET as an antitumor drug.

To conclude, we reported that TET reversed drug resistance through regulating MRP1 not MDR1, and has the potential to be an adjunct to chemotherapy for esophageal cancer. Further studies *in vitro* and *in vivo* are needed to expound the modulation mechanisms, which will provide a better opportunity to exploit TET.

Acknowledgements

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