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Effects of long noncoding RNA (*linc-VLDLR*) existing in extracellular vesicles on the occurrence and multidrug resistance of esophageal cancer cells

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ABSTRACT

Objective: To investigate the relationship between the expression of *linc-VLDLR* in extracellular vesicles (EVs) and esophageal carcinomas development and drug resistance.

Methods: The expression of *linc-VLDLR* and *ABCG2* mRNA in 60 cases of esophageal carcinoma tissue, para-carcinoma tissue and the normal esophagus tissue were detected using Fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR). Fifty percent inhibiting concentration (IC50) of adriamycin (ADM) to Eca109 cells was detected by MTT assay, after the treatment of different concentrations of adriamycin (ADM) on esophageal squamous cell carcinoma Eca109 cell line for 24 h. EVs were extracted from culture medium after the treatment of three concentrations of ADM (setting based on the IC50) on Eca109 cells for 24 h. *Linc-VLDLR* expression in EVs was detected by qRT-PCR. After the treatment of the extracted EVs on virgin Eca109 cells for 48 h, then intervening these cells for 24 h by different concentrations of ADM, the new values of IC50 were detected by MTT assay. Cell cycle, cell apoptosis and ABCG2 protein expression of these Eca109 cells were detected by flow cytometry (FCM). *Linc-VLDLR* and *ABCG2* mRNA expression in these Eca109 cells were detected by qRT-PCR.

Results: Expression of *linc-VLDLR* and *ABCG2* mRNA in esophageal squamous cell carcinoma tissue were significantly higher than that in esophageal atypical hyperplasia and normal esophagus tissue, $P < 0.01$. After the treatment of ADM on Eca109 cells for 24 h, IC50 of Eca109 cells was detected as (0.44 ± 0.02) μg/mL, thus ADM concentrations of 0, 0.2, 0.4 and 0.8 μg/mL were selected to accomplish the following parts of this study. After four groups of Eca109 cells were treated by ADM in different concentrations separately, extracted EVs from the supernatant of all four groups, then labeling these four groups as EVs1, 2, 3 and 4. *Linc-VLDLR* expression in EVs4 was significantly higher than that in EVs1-3, $P < 0.01$. After the treatment of EVs1-4 on virgin Eca109 cells for 48 h, new values of IC50 of Eca109 to ADM were detected by MTT. It was found that the IC50 value of group EVs4 was significantly higher than that of other groups, $P < 0.05$. Flow cytometry results showed that the proliferation index of Eca109 cells in EVs4 was significantly higher than that in EVs1-3 and control groups, $P < 0.01$. Whereas, there was an obviously downward trend in the apoptosis rate of EVs4, compared to other three groups, $P < 0.01$. *Linc-VLDLR* and *ABCG2* mRNA and protein expression level in Eca109 cells of EVs4 group were significantly higher than that of EVs1-3 and control groups, $P < 0.05$.

Conclusions: High expression of *Linc-VLDLR* and *ABCG2* gene in esophageal cancer cells affected the formation of esophageal cancer drug resistance. EVs released by drug-resistant cells were proved that they could upregulate the expression of *ABCG2* in esophageal cancer cells and thus regulate the drug resistance of esophageal cancer cells, which was related to the *linc-VLDLR* carried by EVs.

1. Introduction

Esophageal carcinoma is a relatively common malignant tumour in China, which has high incidence and mortality rates [16]. In the past several decades, the prevention and treatment of esophageal carcinoma raised the concern of the public and posed a serious challenge to the medical services and public health. Routine ways of treatment include surgery, radiotherapy and chemotherapy. Among these, having surgery

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is the preferred alternative, assisted by the other two methods after surgery. To the patients with advanced esophageal carcinoma, there is no necessity of undergoing surgery, thus radiotherapy and chemotherapy change to main treatments. Whereas, the efficacy of chemotherapy often undermines by the toxicity and side-effects of drugs and multidrug resistance generated during this therapy. Therefore, it is considerably important to investigate the mechanism of multidrug resistance generation, in order to find out the way to reverse it.

The ATP binding cassette G2 (ABCG2) is closely related to multidrug resistance of several kinds of tumours [12,17,25,28], and tumour of the esophagus is one of them, proved by our previous studies. However, what has not been discovered is the mechanism of ABCG2-regulating. The long non-coding RNA is also proved to be closely related to the tumours occurrence, development and their multidrug resistance [7,18,27]. In addition, Takahashi et al. [24] indicated that the *linc-VLDLR* in hepatoma cells can affect the drug resistance of these cells, through regulating the expression of ABCG2. It was reported that variable RNAs, microRNAs, long noncoding RNAs and mRNAs can be transported between cells by extracellular vesicles (EVs). EVs are small membranous vesicles secreted from numerous cell types. EVs include exosomes, microvesicles and other types of membrane vesicles. EVs play an important role in intercellular signal transduction, including intercellular multidrug resistance [3,13,22]. In this study, we investigate the relationship between the *linc-VLDLR* and the multidrug resistance of esophageal carcinomas, as well as how the *linc-VLDLR,* existing in EVs and released from multidrug resistant cells, regulates the multidrug resistance of esophageal carcinomas.

2. Materials and methods

2.1. Clinical sample preparation

Specimens were drawn from the biobank at the Fourth Hospital of Hebei Medical University. In total, our study contains 100 surgical specimens, which were resected esophageal carcinomas. Sixty cases of squamous cell carcinoma of the esophagus were randomly selected from these. Those surgeries were carried out between January 2015 and June 2016. Parts taken from every surgical specimen were carcinoma tissue, para-carcinoma tissue (away from the neoplasm boundary about 2–3 cm) and the normal esophagus tissue (away from the neoplasm boundary at least 3–5 cm). Each tissue specimen was divided into two. One was fixed in the 10% neutral buffered formalin, then displayed by paraffin-embedded sections, prepared for HE stain; another was kept in liquid nitrogen, prepared for qRT-PCR. In order to minimize errors, histopathological diagnoses of these tissue specimens were made by two experienced pathologists. 60 cases of squamous cell carcinoma of the esophagus were randomly selected for this study, and correspondingly, the dysplasia and normal tissue relative to these carcinoma tissue were also used in this study. 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. Cell lines

Human esophageal cancer cells Eca109 were obtained from Cancer Institute, the Fourth Hospital of Hebei Medical University, Shijiazhuang, China.

The Eca109 cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Life Technologies, Paisley, Auckland) containing 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Paisley, Auckland) and 100 U/L of penicillin and streptomycin (North China Pharmaceutical Co., Ltd., Shijiazhuang, China) in a humidified atmosphere containing 5% CO_2/95% air at 37 °C.

2.3. Detecting the linc-VLDLR and ABCG2 mRNA expression in different diseased esophageal tissue cells by qRT-PCR

Taking the esophageal tissue stored in the liquid nitrogen and then 1 mL RNA isolater (Vazyme, China) was added to it to extract the total RNA through the one-step method. By following instructions, the total RNA was reversely transcribed to cDNA, and PCR amplification was exerted using cDNA as a template. Additionally, human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and RNU6B were used as the internal reference for standardization. This experiment was carried out according to the standard qRT-PCR procedure, and SYBR-Green I was used as the fluorescent dye. Primers for *ABCG2* were 5′-*GGT CAG AGT GTG GTT TCT GTA GCA*-3′ (forward) and 5′-*GTG AGA GAT CGA TGC CCT GCT TTA*-3′ (reverse), and that for *linc-VLDLR* were 5′-*AGC AGT CAC ATT CAT CGC AC*-3′ (forward) and 5′-*GAG GAA TAG GTG CGA ACT GC*-3′ (reverse). For the internal reference *GAPDH*, primers were 3′-*CAC TAC CGT ACC TGA CAC CA*-5′ (forward) and 3′-*ATG TCG TTG TCC CAC CAC CT*-5′ (reverse), as well as for *RNU6B*, forward primer and reverse primer were 5′-*CTC GCT TCG GCA GCA CA*-3′ and 5′-*AAC GCT TCA CGA ATT TGC GT*-3′ respectively. The relative expression level of *ABCG2* mRNA and *linc-VLDLR* was calculated using the $2^{-\Delta Ct}$ method (ΔCt = CT *ABCG2* – CT *GAPDH*; ΔCt = CT *linc-VLDLR* – CT *RNU6B*).

2.4. Detecting IC50 of Eca109 cells after treatment by ADM for 24 h using MTT method

Monolayer cultured Eca109 cells were digested by 0.25% trypsin, which was used to make the single-cell suspension by adding the RPMI1640 medium with 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Paisley, Auckland) to that mixture. Then, adjusted cell density to 1×10^4 cells /mL and inoculated this suspension to the cell culture plate with 96 wells, before placing the plate into the CO_2 culture chamber (37°, 5% CO_2) in order to achieve cell adherence. When cells were firmly adherent, 24 h later, added in ADM with different concentrations, at 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 μg/mL separately. In each well, the total volume for reaction was set at 200 μl. Cells and the medium was in wells of the negative control group. Only the medium was in wells of the blank control group. Reactions for each ADM concentration, the negative control group and the blank control group were repeated in 3 wells. In the next step, after placing the plate into the culture chamber (37°, 5% CO_2) for 24 h, 20 μl MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO,USA) was added in each well, before putting it again to the chamber for another 4 h. Then, eliminated the liquids to terminating these culturing activities. 180 μl DMSO was added in each well, then inverting or vortexing the mixture for 10 min. Set zero with the blank, and the optical density of each well was read at 490 nm wavelength via a Microplate spectrophotometer. The inhibitory rate (IR) was calculated by the following formula, before calculating the 50% inhibiting concentration (IC50) of cells:

$$IR(\%) = (1\text{-Test group } A_{490} / \text{Control group } A_{490}) \times 100\%$$

2.5. EVs extraction after intervening Eca109 cells by ADM for 24 h

According to the IC50 of Eca109 cells calculated by the last step, three values of ADM concentration were selected, which were 0.2, 0.4 and 0.8 μg/mL. Normal saline with 0 μg/mL ADM was selected using in the control group. The ADM with different concentrations (0, 0.2, 0.4 and 0.8 μg/mL) were applied to Eca109 cells separately. 24 h later, extracellular vesicles (EVs) were collected from the medium via gradient centrifugation, being marked with EVs1, EVs2, EVs3 and EVs4 correspondingly. EVs were collected by the following steps [24]: Firstly, gathered the supernatant obtained by gradient centrifugation (2000 rpm, 10 min; 3000 rpm, 30 min; 11,000 rpm, 1 h). Then,

centrifuged this supernatant with the speed of 110,000 × g lasting 16 h. After disposing of the supernatant, gained the deposit of EVs from dissolving the remainings through PBS. Lastly, quantified EVs by Nanodrop (Thermo, USA) and preserved them in many pieces, under −80°.

2.6. Detecting the expression of linc-VLDLR in EVs by qRT-PCR

This process was guided by one-step method. Considering the collected EVs1 to EVs4 as four clusters, total RNA of each EVs cluster was extracted by adding 1 mL RNA isolater (Vazyme, China) to extract the RNA of EVs. Reverse transcription of total RNA to cDNA was applied following instructions, and then the generated cDNA was used as a template in PCR amplification. Additionally, primers for *linc-VLDLR* were 5′-*AGC AGT CAC ATT CAT CGC AC*-3′ (forward) and 5′-*GAG GAA TAG GTG CGA ACT GC*-3′ (reverse), and that for the internal reference *RNU6B* were 5′-C*TC GCT TCG GCA GCA CA*-3′ (forward) and 5′-*AAC GCT TCA CGA ATT TGC GT*-3′ (reverse). The $2^{-\Delta Ct}$ method was used to calculate the relative expression level of *linc-VLDLR* (ΔCt = CT *linc-VLDLR* − CT *RNU6B*).

2.7. Intervening Eca109 cells by EVs

The monolayer cultured Eca109 cells were digested by 0.25% trypsin, and then were cultured in the RPMI1640 medium (Gibco-BRL, Life Technologies, Paisley, Auckland) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Paisley, Auckland), to produce the single-cell suspension. Cell density of this suspension was adjusted to 1×10^5 cells /mL and seeded this suspension into 6-well culture plates. Put these plates at 37° in a humidified atmosphere with 5% CO_2 for 48 h. Next, five groups were specified, and each group repeated 3 wells. Added EVs(1–4) and normal saline to these five groups respectively, which were marked with EVs1, EVs2, EVs3, EVs4 and control group. Concentration of all EVs solutions was 50 μg/mL. Same amount of solution to each well was 50 μL, no matter it was EVs or normal saline. 48 h later, collected cells cultured in these five groups, and then adjusted the cell density of single-cell suspension to 1×10^6 cells /mL.

2.7.1. Detecting expression of ABCG2 mRNA and linc-VLDLR in cells by qRT-PCR

Took 1 mL single-cell suspension prepared by the last step (see Chapter 2.7). After washing cells by the ice-cold PBS, added 1 mL RNA isolater (Vazyme, China) to extract total RNA. Reverse transcription of total RNA to cDNA was carried out for PCR amplification. On the basis of real-time PCR processes, SYBR-Green I was used as fluorescent dye. Repeat these PCR processes 3 times for each group. Furthermore, primers for *ABCG2* were 5′-*GGT CAG AGT GTG GTT TCT GTA GCA*-3′(forward) and 5′-*GTG AGA GAT CGA TGC CCT GCT TTA*-3′(reverse), and that for *linc-VLDLR* were 5′-*AGC AGT CAC ATT CAT CGC AC*-3′ (forward) and 5′-*GAG GAA TAG GTG CGA ACT GC*-3′ (reverse). Primers for internal reference *GAPDH* were 3′-*CAC TAC CGT ACC TGA CAC CA*-5′ (forward) and 3′-*ATG TCG TTG TCC CAC CAC CT*-5′ (reverse), and that for internal reference *RNU6B* were 5′-*CTC GCT TCG GCA GCA CA*-3′ (forward) and 5′-*AAC GCT TCA CGA ATT TGC GT*-3′ (reverse). Relative expression levels of *ABCG2* mRNA and *linc-VLDLR* were both calculated by the $2^{-\Delta Ct}$ method (ΔCt = CT *ABCG2* − CT *GAPDH*; ΔCt = CT *linc-VLDLR* − CT *RNU6B*).

2.7.2. Detecting expression of ABCG2 protein in cells by flow cytometry

One mL single-cell suspension, prepared in Chapter 2.7, was taken from each group. For each group, before 100 μL PBS was added to make cells suspended, cells were washed by the ice-cold PBS. The FITC-conjugated anti-ABCG2 (5D3) antibody (Biolegend, San Diego, CA, USA) was added and cells were incubated at room temperature for 30 min in the dark. The labeled cells were washed in PBS, centrifuged for 5 min at 1200 x g and stored at 4 °C until use. The stained cells were analyzed using Beckman Coulter FC-500 type flow cytometer (Beckman Coulter, Miami, FL, USA). The mean fluorescence intensity represents the expression of ABCG2 protein.

2.7.3. Detecting IC50 of Eca109 cells that had been intervened by EVs, after these cells were intervened by ADM through MTT method

Seeded the single-cell suspension of five groups respectively, prepared in Chapter 2.7, into 96-well culture plates. Carried out experiments as described in Chapter 2.4 and calculated IC50 of Eca109 cells from each group after the intervention by ADM for 24 h.

2.7.4. Cell cycle detected by flow cytometry (FCM)

One mL single-cell suspension, prepared in Chapter 2.7, was taken from each group. Washed cells by the ice-cold PBS, and fixed cells by 70% alcohol at 4 ° for 24 h. Then, after washing cells twice by the ice-cold PBS, added 100 μL PBS to each group to make cells suspended in. This mixture was added in 1 mL propidium iodide (BD, San Diego, CA, USA) for staining, at 4° lasting 30 min, before being detected by the flow cytometer. Then, cell proliferation cycle was detected by flow cytometry (Beckman Coulter, Miami, FL, USA) and analyzed by the Muticycle AV software (Beckman Coulter, Miami, FL, USA). The proliferation index (PI) was calculated according the following formula: $PI = (S + G_2/M)/ (G_{0/1} + S + G_2/M) \times 100\%$.

2.7.5. Eca109 cell apoptosis detected by FCM

One mL single-cell suspension, prepared in Chapter 2.7, was taken from each group. For each group, before 100 μL PBS was added to make cells suspended in, cells were washed by the ice-cold PBS. The cells were stained with propidium iodide and annexin V-fluorescein isothiocyanate (FITC) (Beckman Coulter, Miami, FL, USA), and analyzed with Beckman Coulter FC-500 type flow cytometer (Beckman Coulter, Miami, FL, USA). Early apoptotic cells tested positive for annexin V and negative for propidium iodide staining, whereas late apoptotic cells undergoing secondary necrosis were positive for both annexin V and propidium iodide staining.

2.8. Statistical analysis

All data were presented as means ± standard deviation (SD). Comparison of means from multiple samples was done by one-way ANOVA analysis. Paired comparison was exerted using *LSD-t* test. $P < 0.05$ was considered as significant difference. Statistical analysis in this research was entirely carried out by the SPSS11.5 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Detecting the linc-VLDLR and ABCG2 mRNA expression in different diseased esophageal tissue cells by qRT-PCR

As shown in Fig. 1, the expression levels of *linc-VLDLR* and *ABCG2* mRNA in esophageal cancer tissues were significantly higher than that in the normal esophagus tissues and atypical dysplasia tissues, $P < 0.01$.

3.2. Detecting IC50 of Eca109 cells after being intervened by ADM for 24 h using MTT method

After treatment of ADM for 24 h, the IC50 of Eca109 cells was (0.44 ± 0.02) μg/mL. Based on this value, three concentrations of ADM (0.2, 0.4 and 0.8 μg/mL) were separately used to intervene Eca109 cells to generate EVs.

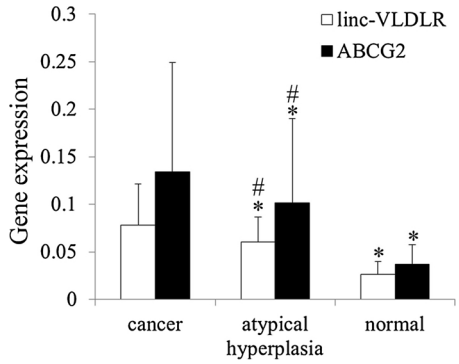

Fig. 1. Expression of linc-VLDLR and ABCG2 mRNA in different esophageal tissue cells.
* P < 0.01, compared with cancer group;# P < 0.01, compared with normal group. Linc-VLDLR and ABCG2 mRNA expression in cancer group were significantly higher than that in atypical hyperplasia and normal group, P < 0.01.

3.3. Detecting the expression of linc-VLDLR in EVs by qRT-PCR

There was an upward trend in *linc-VLDLR* expression from EVs1 to EVs4. Comparison among groups had statistical significance ($P < 0.01$); the *linc-VLDLR* in EVs4 group was significantly higher than that in EVs1, EVs2 and EVs3 groups. (see Fig. 2).

3.4. Detecting IC50 of Eca109 cells that had been intervened by EVs, after these cells were intervened by ADM through MTT method

As exhibited in Fig. 3, IC50 values of EVs1 to EVs4 were all higher than that of the control group, $P < 0.01$ (see Fig. 3). Particularly, IC50 of EVs2 was twice higher than that of the control group. IC50 of EVs3 and EVs4 were almost three times as high as IC50 of the control group (see Fig. 3). In addition, IC50 of EVs4 was much higher than that of EVs1 to EVs3, $P < 0.05$ (see Fig. 3).

3.5. Cell cycle detected by FCM

To cell proliferation index, all EVs groups were higher than control group ($P < 0.01$), and among these, EVs4 group was the highest ($P < 0.01$) (see Fig. 4). To $G_{0/1}$ of cell cycle, EVs groups were all lower than control group ($P < 0.01$), and EVs 4 was the lowest compared to other EVs groups ($P < 0.01$) (see Fig. 4).

3.6. Cell apoptosis detected by FCM

Four EVs groups all had lower apoptosis rate of Eca109 cells, compared with the control group ($P < 0.01$) (see Fig. 5). Apparently, there was a decrease trend in apoptosis rate from EVs1 to EVs4, and EVs4 had the lowest value ($P < 0.01$) (see Fig. 5).

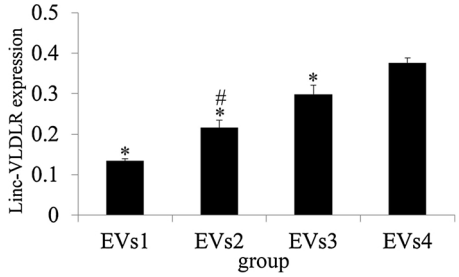

Fig. 2. Linc-VLDLR expression in EVs was detected by qRT-PCR.
* P < 0.01 compared with the EVs4 group; # P < 0.01 compared with the EVs1 and EVs3 groups. The linc-VLDLR expression in EVs 4 group was significantly higher than that in other groups (EVs1-EVs3), P < 0.01.

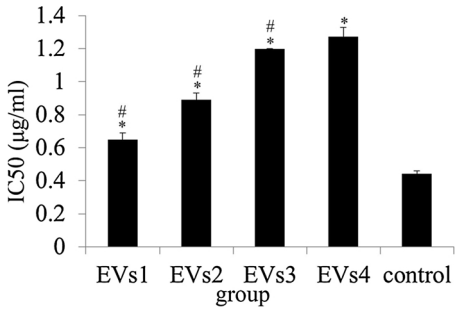

Fig. 3. IC50 of ADM on Eca109 cells after the treatment with EVs.
* P < 0.01 compared with the control group; # P < 0.05 compared with the EVs4 group. The IC50 of ADM on Eca109 cells was increased after the treatment with EVs.

3.7. Expression of linc-VLDLR and ABCG2 mRNA in Eca109 cells after treatment with EVs

Considerably, expression of *ABCG2* mRNA in Eca109 cells of the four groups (EVs1-4) were generally higher than that of control group ($P < 0.01$) (see Fig. 6). In addition, expression of *linc-VLDLR* in Eca109 cells of groups EVs2-4 were higher than that of control group ($P < 0.01$) (see Fig. 6), whereas there was no statistical significance, comparing expression of *linc-VLDLR* of EVs1 with that of control group ($P > 0.05$). To EVs4, expression of both *ABCG2* mRNA and *linc-VLDLR* in Eca109 cells were significantly higher than that of the other three EVs groups (EVs1-3) and also the control group ($P < 0.05$) (see Fig. 6).

3.8. Expression of ABCG2 protein in Eca109 cells after treatment with EVs

ABCG2 protein expression in Eca109 cells of the four groups (EVs1-4) were generally higher than that of control group ($P < 0.05$) (see Fig. 7). To EVs4, expression of ABCG2 protein in Eca109 cells was significantly higher than that of the other three EVs groups (EVs1-3) and also the control group ($P < 0.05$) (see Fig. 7). The ABCG2 protein expression of Eca109 cells was consistent with the *ABCG2* mRNA expression after the treatment of EVs.

4. Discussion

Esophageal carcinomas is a common malignant epithelial tumour with high incidence and mortality ratio [30]. Chemotherapy is a significant treatment for patients who have advanced disease and are hardly to be cured by surgery. Whereas, efficacy of chemotherapy is usually undermined by multidrug resistance. Many studies indicate that it is the microenvironment that plays an important role in tumours occurrence, development and multidrug resistance [9,11,14]. Tumour cells are able to create a tumour-growing benefit microenvironment through intercellular information transmission [15,23,29], in other words, indicating the seed-soil theory. Therefore, studies on cells stress response, such as the acquired drug-resistance caused by microenvironment changing, provide a new direction for esophageal carcinomas treatment.

EVs are ultrastructure of vesicles generated and released when cells are suffering from stress. EVs are one of the bioactivators secreted paracellular, containing microvesicles (MVs) and exosomes [21]. EVs has a globular membranous structure surrounded externally by lipid bilayer, of which the diameter fluctuates from 40 to 5000 nm A variety of cells that are able to release EVs to their living microenvironment include multiple hemopoietic system cells (platelet, megakaryocyte, monocyte, T-lymphocyte and B-lymphocyte etc.), tumour cells, mesenchymal stem cells, embryonic stem cells and alveolar epithelial cells etc [1,6,19]. EVs can also be detected in cell culture fluid, serum, saliva, malignant ascites, milk and urine [10,20,26].

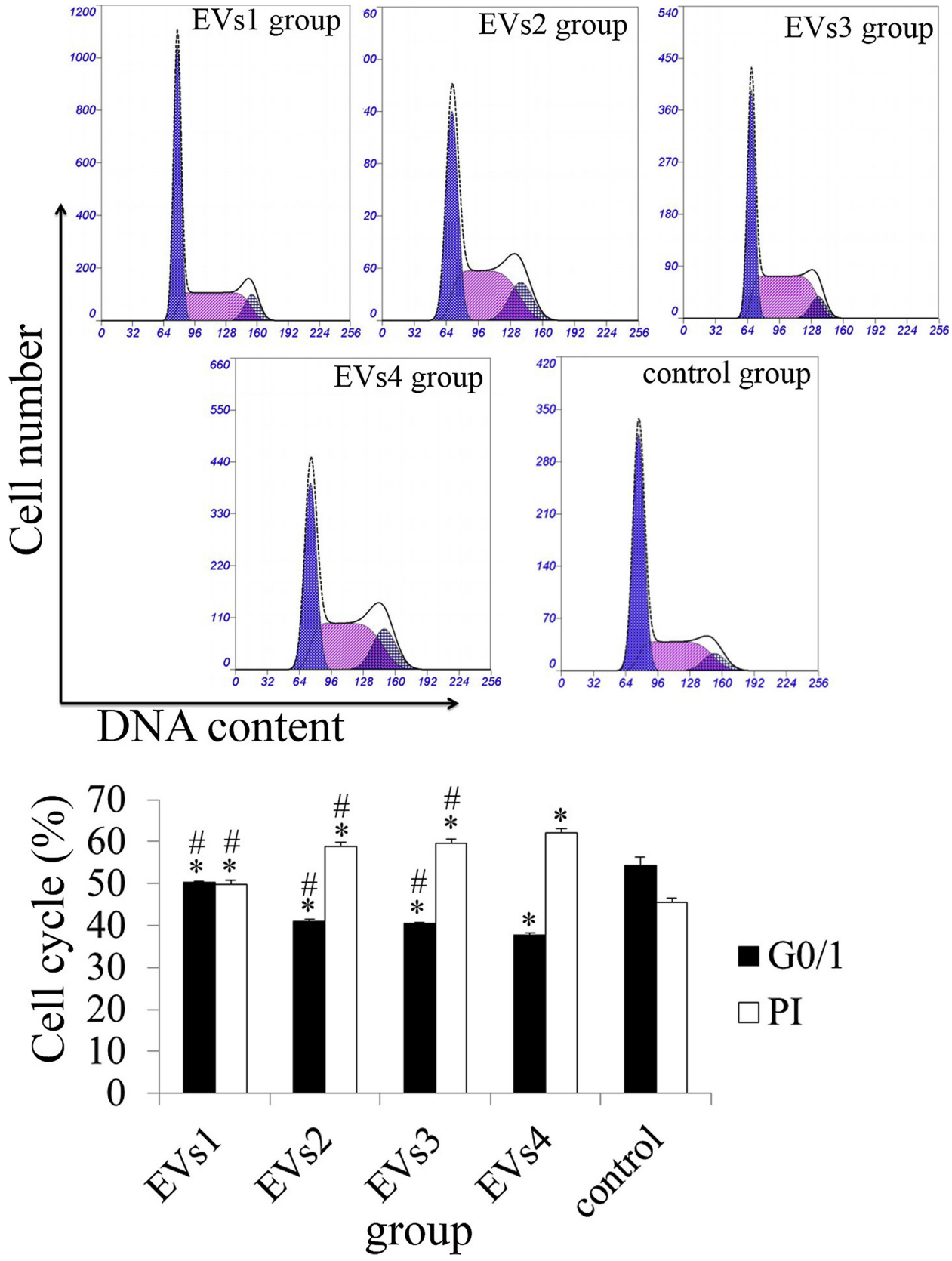

Fig. 4. Cell cycle of Eca109 cells was detected by flow cytometry.
* $P < 0.01$ compared with the control group; # $P < 0.01$ compared with the EVs4 group. To cell proliferation index, all EVs groups were higher than control group ($P < 0.01$).

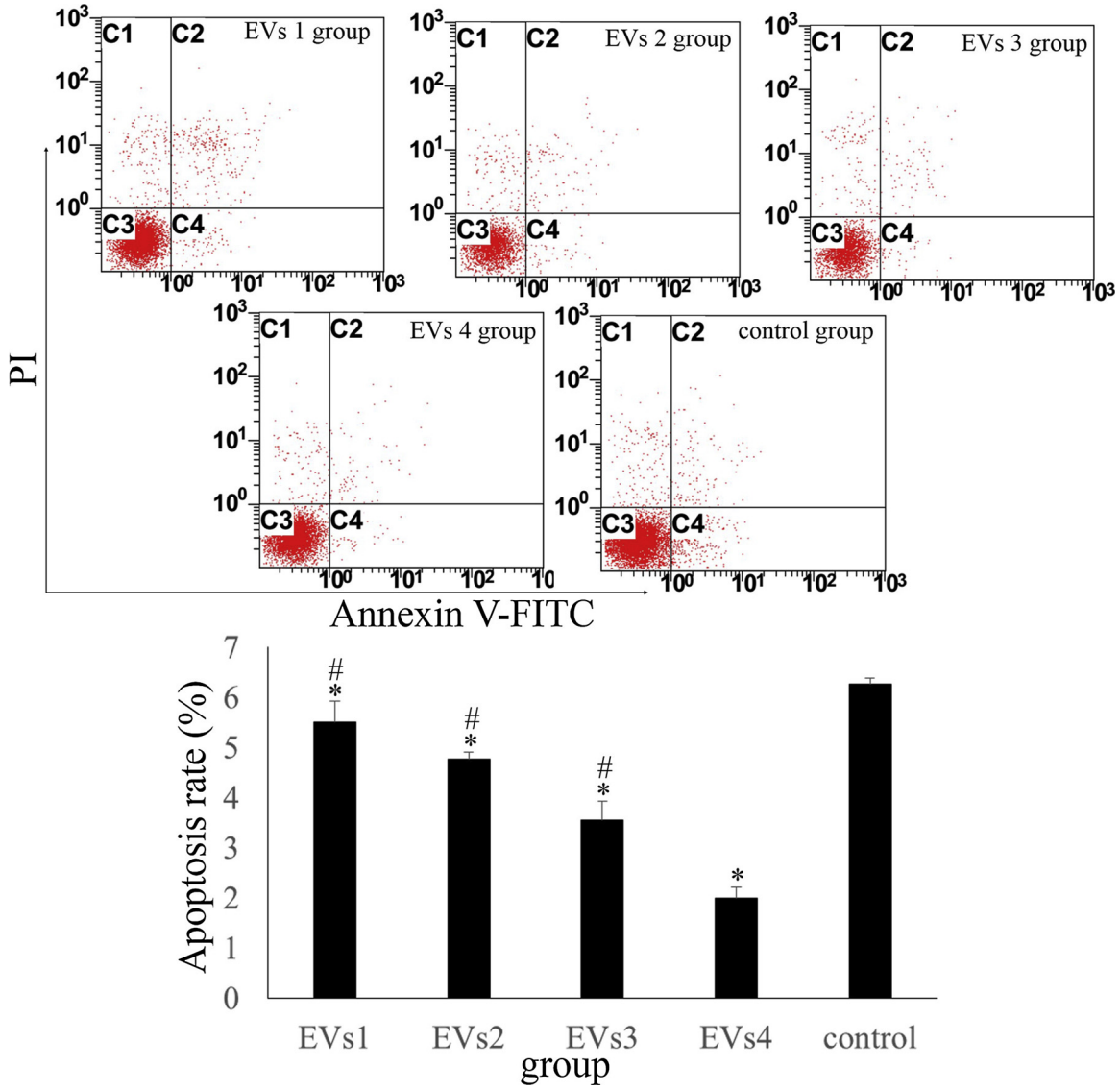

Fig. 5. Cell apoptosis rate of Eca109 cells was detected by flow cytometry.
* P < 0.01 compared with the control group; # P < 0.01 compared with the EVs4 group. Four EVs groups all had lower apoptosis rate of Eca109 cells, compared with the control group (P < 0.01). The cell apoptosis rate of Eca109 cells was decreased after the treatment with EVs.

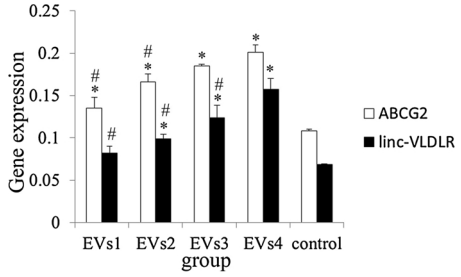

Fig. 6. Linc-VLDLR and ABCG2 gene expression of Eca109 cells after the treatment with EVs.
* P < 0.01 compared with the control group; # P < 0.01 compared with the EVs4 group. Linc-VLDLR and ABCG2 mRNA of Eca109 cells were significantly increased after the treatment with EVs.

When EVs generate, various signaling molecules sauced from parent cells will concentrate in, including protein, mRNA and non-coding RNA etc. These signaling molecules are going to be released to target cells after the interaction between EVs and target cells, in order to change phenotype and genotype of target cells [2,5]. Studies show that EVs released by certain tumour cells stimulate cell proliferation, angiogenesis and metastasis and immune escape, via affecting tumour cells, endothelial cells, tumour-associated fibroblast and immune cells existing in EVs' microenvironment [4,8]. As a result, tumour formation and growth are also be facilitated. Takahashi et al. [24] demonstrates that the EVs secreted by hepatoma carcinoma cells can regulate the expression of *ABCG2* in target cells via *linc-VLDLR*, in order to induce the acquired drug-resistance of target cells (hepatoma carcinoma cells). Our previous studies found that *ABCG2* was closely related to the multidrug resistance of esophageal carcinomas, whereas the regulation mechanism of *ABCG2* had not been clarified. Therefore, taking the clue that EVs transmit information intercellularly in the tumour microenvironment into consideration is significant to prevent the multidrug resistance of tumours. This study aims to explore esophageal carcinomas' multidrug resistance mechanisms regulated by EVs released from drug-resistant esophageal carcinoma cells; to investigate effects of the EVs—*linc-VLDLR*—*ABCG2* pathway in the formation mechanism of esophageal carcinomas multidrug resistance; to develop updated insights in preventing esophageal carcinoma multidrug resistance; to provide the experimental foundation for the clinical treatment.

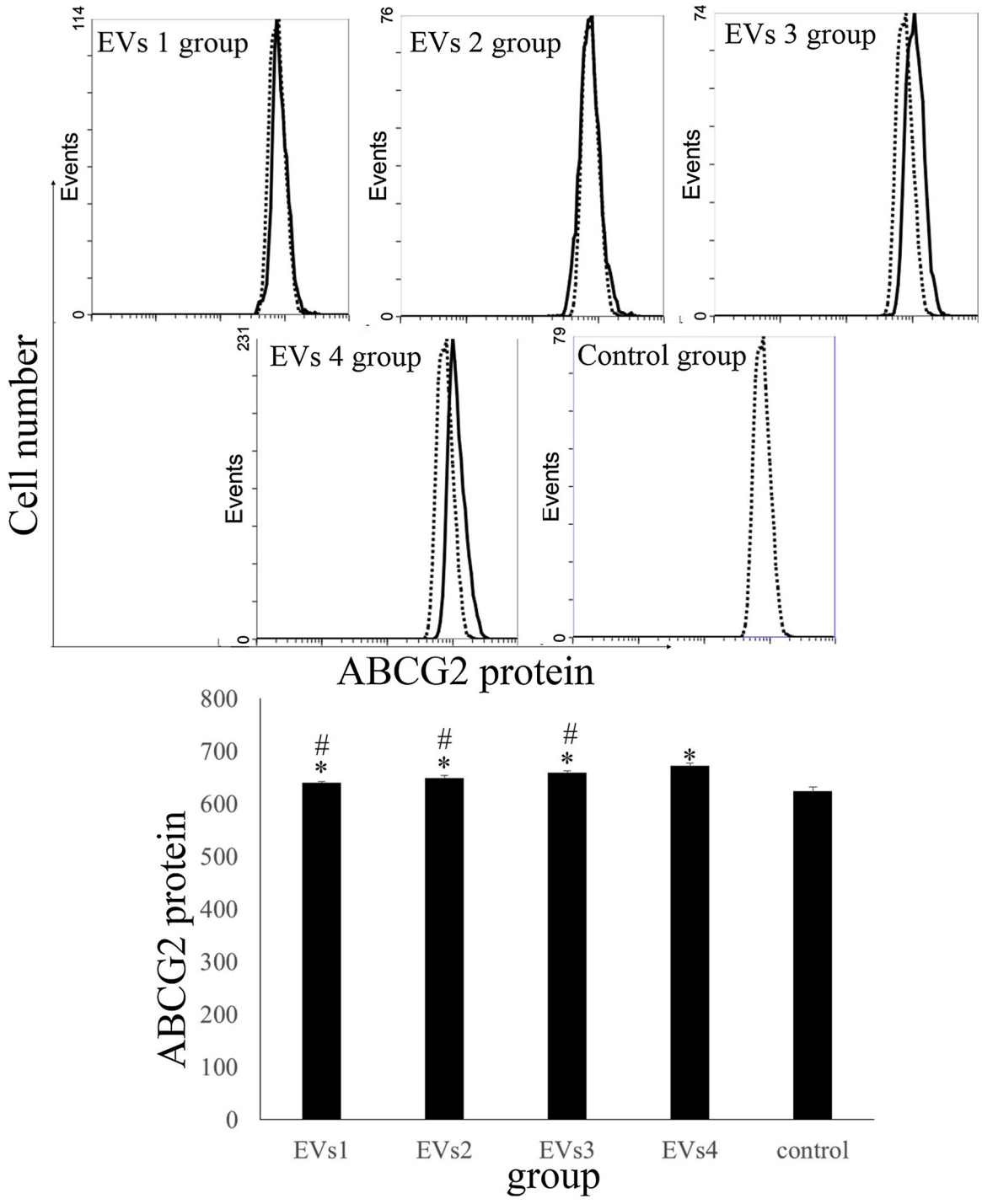

Fig. 7. Expression of ABCG2 protein in Eca109 cells after treatment with EVs.
ABCG2 protein expression in Eca109 cells was increased after the treatment with EVs. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the EVs 4 group.

In this study, we used different concentrations of ADM to intervene the Eca109 cells for 24 h, in order to have transient drug-resistant cells, and to collect EVs released by these cells. Expression level of *linc-VLDLR* in these EVs released by Eca109 cells treated with ADM was considerable higher than that in the EVs released by control group, and increased with the increasing ADM concentration. Comparing the Eca109 cells intervened by EVs released from experimental groups for 48 h with Eca109 cells in control group, it was found that in experimental groups, drug resistance of cells to ADM were induced, and IC50 value of each group increased significantly; apoptosis rate of cells in each group decreased; expression levels of *linc-VLDLR* and *ABCG2* in cells got higher; proliferation index of cells in each group rose evidently, while in each group the proportion of $G_{0/1}$ stage decreased, dependent on ADM concentration. Expression of *linc-VLDLR* in EVs released from Eca109 cells intervened by high concentration ADM was obviously higher than that from Eca109 cells intervened by low concentration ADM and NS. The growth in expression levels of *linc-VLDLR* and *ABCG2* in Eca109 cells was caused by the intervention of EVs with high expression level of *linc-VLDLR*. In Eca109 cells, the high expression level of *ABCG2* resulted in the multidrug resistance of cells.

Results illustrated that high expression levels of *linc-VLDLR* and *ABCG2* were closely related to the multidrug resistance of esophageal carcinomas. *Linc-VLDLR* carried by EVs, which were released by the drug-resistant esophageal carcinoma cells, could cause the acquired drug-resistance of target cells through regulating the expression of *ABCG2* in target cells. This study investigated the EVs—*linc-VLDLR*—*ABCG2* pathway that were able to cause the esophageal carcinoma multidrug resistance, in order to provide new perspectives on esophageal carcinoma multidrug resistance and to provide the experimental foundation for the clinical treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

Not applicable.

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