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Original Article

Cancer stem-like cells contribute to paclitaxel resistance in esophageal squamous cell carcinoma

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Abstract: Objective: To examine the role of esophageal squamous cell carcinoma (ESCC) stem cells in paclitaxel resistance through the molecular characterization of ESCC stem cells. Methods: A resistant cell line (RR-ECl09) of cells were established using intermittent induction and time increments of high-dose paclitaxel in a human esophageal squamous cell carcinoma line (EC109). The multidrug resistance of RR-ECl09 cells to anticancer agents was evaluated by MTT assay. The RR-EC109 and EC109 cells were used for sphere formation assays, clonogenicity assays, stem cell gene expression, and the expression of epithelial-mesenchymal transition markers. Results: The RR-EC109 cells were established over 7 months. RR-ECl09 cells had 67.258 fold resistance to paclitaxel. The percentage of sphere formation and clone proliferation ability of RR-EC109 cells was higher than that of EC109 cells ($P < 0.05$). The amount of side population cells in RR-EC109 cells was higher than that of EC109 cells ($P < 0.05$). RR-EC109 cells produced more mRNA for Bmi1, Nanog, Oct4, Sox2, ABCG2, Nestin, and Ki-67 than EC109 cells ($P < 0.05$). E-cadherin expression was lower in RR-EC109 cells than in EC109 cells, while N-cadherin, Snail, and Twist expressions were higher in RR-EC109 cells than in EC109 cells ($P < 0.05$). Conclusions: Cancer stem cell (CSC)-like cells exist among paclitaxel-resistant cells in ESCC and may play a role in ESCC drug resistance.

Keywords: Cancer stem-like cells, esophageal squamous cell carcinoma, paclitaxel, resistance

Introduction

Esophageal cancer is common worldwide and represents a significant global health burden. Esophageal cancer ranks seventh in terms of incidence (572,034 new cases) and sixth in mortality overall (508,585 deaths) in 2018 [1]. China alone accounts for approximately 50% of esophageal cancer cases worldwide. Squamous cell carcinoma accounts for roughly 90% of all esophageal cancers [2]. Moreover, the prognosis of cancer sufferers remains bleak. Patients with esophageal squamous cell carcinoma report a 5-year survival rate of about 20% [3]. Chemotherapy, radiation, and surgery are recognized as standard treatment options. Paclitaxel is a commonly used chemotherapeutic drug for esophageal squamous cell carcinoma (ESCC).

However, paclitaxel resistance significantly impacts its efficacy and can even result in chemotherapy failure. The underlying molecular processes involved in this chemoresistance remain largely unknown. A more detailed understanding of the molecular mechanisms by which tumor cells survive chemotherapy will likely lead to novel therapeutic targets and more successful outcomes.

According to the cancer stem cell theory, a small number of cells in malignancies have similar activities to normal tissue stem cells and can self-renew and differentiate into daughter cells for an extended period. Cancer stem-like cells (CSCs), also known as cancer-initiating cells (CICs), are a subset of these cells. The cancer stem cell theory was initially presented in 1959 [4]. CSCs have been found in breast cancer, liver cancer, brain tumors, lung cancer, and other malignancies in subsequent research [5-8]. According to the cancer stem cell hypothesis, CSCs may be the root cause of tumorigenicity, recurrence, and metastasis [9, 10].

Furthermore, CSCs appear to be extremely resistant to various chemotherapies [11, 12]. To date, the distribution and properties of ESCC stem-like cells are undetermined. Furthermore, there is no direct evidence that paclitaxel resistance is caused by esophageal CSCs. Understanding the molecular underpinnings underlying CSC self-renewal and differentiation, which are likely associated with tumor progression, is becoming increasingly relevant. The objectives of this study are to characterize ESCC stem cells molecularly and investigate the function of ESCC stem cells in paclitaxel resistance.

Materials and methods

Cell culture and drug selection

Shanghai Biological Cell Bank provided the human esophageal cancer cell lines EC109 (Shanghai, China). EC109 cells were grown in RPMI-1640 media (Gibco, USA) containing 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, and 100 mg/l streptomycin, and incubated at 37°C in a 5% CO_2 atmosphere. Cells were monitored every 24 hours and passaged every two days. Logarithmic phase cells were selected for the studies.

EC109 cells were treated for 2 hours with 0.625 μg/ml paclitaxel (Hainan General Kangli, China) and rinsed three times with phosphate buffer saline (PBS). The culture medium was added to continue the cell culture process. The live cells were amplified to 80-90% cell density after 24 hours and subcultured for three generations. The drug was applied to the cells twice, as described above. Then the drug induction time was increased to 4 h, and the above experimental steps were repeated 3 times, whereafter the drug-resistant cells (DRCs) RR-EC109 were obtained.

Drug resistance stability test

RR-EC109 and EC109 cells were inoculated into 96-well plates at a density of 7×10^3 cells per well. After 24 hours, 100 μl of paclitaxel in 8 concentration gradients (0 μg/ml, 0.0125 μg/ml, 0.025 μg/ml, 0.05 μg/ml, 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 10 μg/ml) was introduced to each well in the experimental group, with 6 parallel holes for each concentration gradient. Each well in the control group received 100 μl of culture media. After 48 hours, each well received a 20 μl methylthialazole tetrazolium (MTT) solution and was cultured for 4 hours. Each well received 150 μl of dimethyl sulfoxide and was shaken for 10 minutes. The optical density at 490 nm was determined using an enzyme-linked immunosorbent assay. The preceding procedures were then repeated on the 30th, 60th, 90th, and 120th day after withdrawal.

Sphere formation assays

In ultra-low-attachment 6-well plates, RR-EC109 and EC109 cells were inoculated at a density of 5000 cells/well. Every two days, half of the pelletizing culture media (DMEM/F12 culture medium with 20 ng/ml epidermal growth factor, 10 ng/ml fibroblast growth factor, and 1% B27) was changed. Colonies with > 20 cells were counted after 10 days in culture.

Clonogenicity assays

RR-EC109 and EC109 cells were inoculated into cell culture flasks with 600 cells/bottle (3 bottles each) and incubated at 37°C in a 5% CO_2 atmosphere. Every two days, the culture media was replaced. After 10 days, the cells were fixed in 100% methanol for 5 minutes, and adherent cells were stained with 0.5% crystal violet for a further 5 minutes. The number of cloned cells with > 50 cells were counted.

Side population analysis

RR-EC109 and EC109 cells were cultivated to 90% density in the culture flask. Cells were digested with 0.25% trypsin before being centrifuged for 5 minutes, washed twice with PBS, and then resuspended in RPMI-1640 culture at a concentration of 1×10^6 cells/ml. Single-cell suspensions of RR-EC109 and EC109 were stained with Hoechst 33342 (Sigma, United States) at a final concentration of 5 μg/ml and incubated for 90 minutes at 37°C in the dark with interval mixing. Before adding Hoechst 33342, a negative control was incubated for 30 minutes at 37°C with 50 uM verapamil (Sigma, United States). After illumination with a 355 nm UV light, fluorescence-activated cell sorting (FACS) examination was carried out using 450 nm (Hoechst blue) and 660 nm (Hoechst red) filters.

Table 1. Polymerase chain reaction primer sequences

Gene product	Forward (F) and reverse (R) primers (5' to 3')	Size (bp)
Bmi1	AAATGCTGGAGAACTGGAAAG	124
	CTGTGGATGAGGAGACTGC	
Nanog	ATTCAGGACAGCCCTGATTCTTC	76
	TTTTTGCGACACTCTTCTCTGC	
Oct4	GTGGAGAGCAACTCCGATG	86
	TGCTCCAGCTTCTCCTTCTC	
Sox2	CGAGTGGAAACTTTTGTCGGA	74
	TGTGCAGCGCTCGCAG	
ABCG2	CACGTGATTCTTCCACAAGCC	74
	CATGTACTGGCGAAGAATATTTGGT	
Nestin	AACTGGCACACCTCAAGATGT	235
	TCAAGGGTATTAGGCAAGGGG	
Ki-67	CAGTACTCGGAATGCAGCAA	170
	CAGTCTTCAGGGGCTCTGTC	
E-cadherin	ATTCTGATTCTGCTGCTCTTG	400
	AGTAGTCATAGTCCTGGTCTT	
N-cadherin	CTCCTATGAGTGGAACAGGAACG	121
	TTGGATCAATGTCATAATCAAGTGCTGTA	
Snail	GAGGCGGTGGCAGACTAG	159
	GACACATCGGTCAGACCAG	
Twist	CGGGAGTCCGCAGTCTTA	130
	TGAATCTTGCTCAGCTTGTC	
GAPDH	AATTGAGCCCGCAGCCTCCC	153
	CCAGGCGCCCAATACGACCA	

Stem cell gene expression

Total RNA was isolated from RR-EC109 and EC109 cells. An ultraviolet spectrophotometer was used to measure the concentration and purity of RNA, and gel electrophoresis was used to determine the integrity of the RNA. The target cDNA was amplified using primers for the Bmi1, Nanog, Oct4, Sox2, ABCG2, Nestin, and Ki-67 genes (**Table 1**) (All primers were produced by Beijing Ook Biological Co. (Beijing, China)). To quantify PCR reactions, combine 12.5 μl 2× SYBR green I master mix, 8.5 μl ddH_2O, 2 μl cDNA, and 1 μl 10 mM forward chain and 1 μl 10 mM reverse chain. The reaction conditions are as follows: 95°C predegeneration 30 sec; 95°C denaturation 5 sec; 60°C annealing 30 sec (denaturation and annealing repeated 45 times); final 72°C extends 33 sec. Then, the melting curve was immediately analyzed, 95°C 15 sec, 60°C 30 sec, from 65°C, with each increase of 0.5°C kept 15 sec, gradually increased to 95°C. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Expression of markers of epithelial-mesenchymal transition

Total RNA was extracted from RR-EC109 and EC109 cells. The concentration, purity, and integrity of the RNA were assessed. The target cDNA was amplified using primers for the E-cadherin, N-cadherin, Snail, and Twist genes after RNA was reverse transcribed into cDNA (**Table 1**). To quantify PCR reactions, combine 12.5 μl 2× SYBR green I master mix, 8.5 μl ddH_2O, 2 μl cDNA, and 1 μl 10 mM forward chain and 1 μl 10 mM reverse chain. The reaction conditions and post-reaction dissolution curve analyses are identical to those mentioned above.

Statistical methods

The results were analyzed by SPSS 19.0 software. Data are expressed as mean ± standard deviation. Student's t-tests were used for the comparison of two groups. $P < 0.05$ was considered significant.

Results

Isolation of paclitaxel-resistant ESCC cells

The RR-EC109 cells were induced over 7 months using high dosage intermittent induction and time increment measurements. The majority of the cells perished after the administration of paclitaxel, although a few cells remained attached to the wall. These cells were smaller, had varying sizes and uneven shapes, and proliferated in clusters. Paclitaxel treatment duration was extended in the second stage, whereby cell adaptability was improved, the number of dead cells was greatly reduced, and the recovery of proliferation was expedited.

The stability of drug resistance of RR-EC109 cells was detected by the MTT method. The half-maximal inhibitory concentration (IC50) of

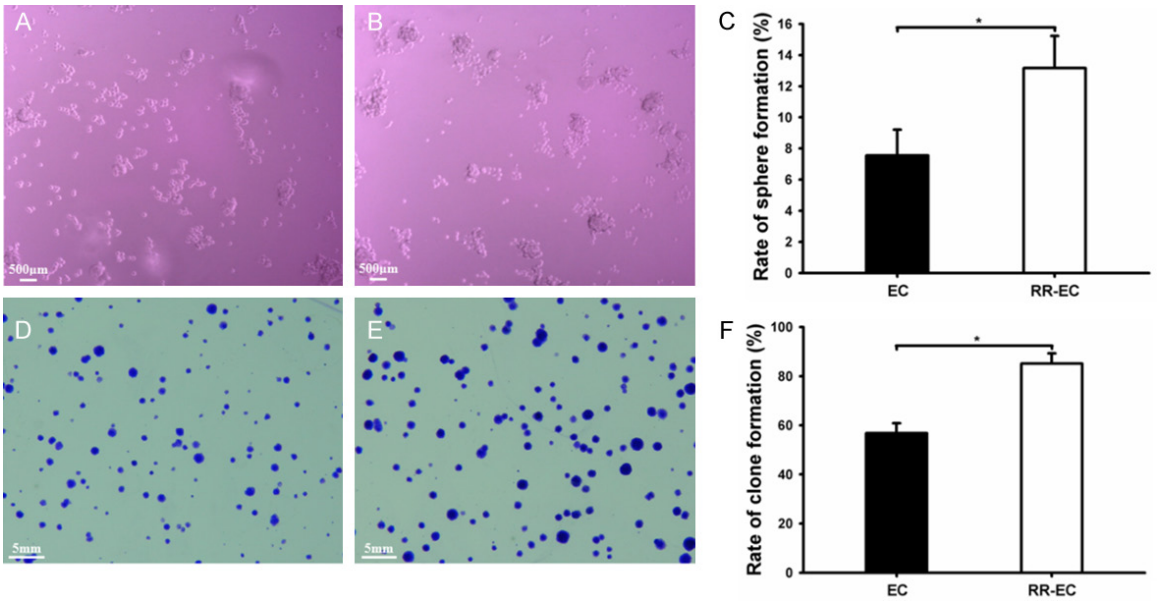

Figure 1. RR-EC109 cells display higher sphere formation and clone formation capacity. A. Primary tumor spheres originating from EC109 cells (×4 objective). B. Primary tumor spheres originating from RR-EC109 cells (×4 objective). C. Statistical analysis of sphere formation efficiency. *P < 0.01. D. Clone-forming capacity (> 50 cells/clone) of EC109 cells. E. Clone-forming capacity (> 50 cells/clone) of RR-EC109 cells. F. Statistical analysis of the clone formation efficiency. *P < 0.05.

EC109 cells was (0.068±0.003) μg/ml, and the IC50 of RR-EC109 cells was (3.578±0.089) μg/ml ($P < 0.001$). The resistance index (RI) of RR-EC109 cells to paclitaxel was 67.258, which denotes high resistance. The IC50 and RI of RR-EC109 cells to paclitaxel were around 3.5 μg/ml and 67 on the 30th, 60th, 90th, and 120th day after withdrawal, indicating that the drug resistance of RR-EC109 cells was robust.

RR-EC109 cells display self-renewal capacity

In an ultra-low attachment and serum-starved culture environment, we tested the capacity of EC109 and RR-EC109 cells to form spherical colonies. Spherical colonies were counted after 10 days of cultivation. The RR-EC09 cells had nearly twice as many spherical colonies as the EC109 cells (13.2±2.1% for the RR-EC109 cells vs. 7.6±1.7% for the EC109 cells) ($P < 0.01$) (**Figure 1**).

In order to determine the clonogenic capacity of EC109 and RR-EC109 cells, colonies were counted. Clones were produced by the cells at a rate of 36.8±3.9% for EC109 cells and 65.2±4.1% for RR-EC109 cells ($P < 0.05$) (**Figure 1**).

RR-EC109 cells contain a larger side population

In both EC109 and RR-EC109 cells, we measured the side population (SP) fraction. A larger percentage of SP cells was identified when RR-EC109 cells were compared to EC109 cells. According to the flow cytometric study, the percentage of SP cells rose from 2.3% in EC109 cells to 5.8% in RR-EC109 cells ($P < 0.05$). These findings showed that CSCs may be enhanced in the context of drug selection. There were essentially no SP cells in RR-EC109 and EC109 cells in the negative control group (pre-added verapamil), which were 0.00% and 0.04%, respectively, these were significantly different from those without verapamil ($P < 0.05$) (**Figure 2**).

RR-EC109 cells express stem cell markers

The expression of stem cell genes was examined to validate the RR-EC109 cells' stem cell characteristics. The quantification PCR analysis revealed that RR-EC109 cells produced more mRNA for Bmi1, Nanog, Oct4, Sox2, ABCG2, Nestin, and Ki-67 than EC109 cells (all $P < 0.05$) (**Figure 3**).

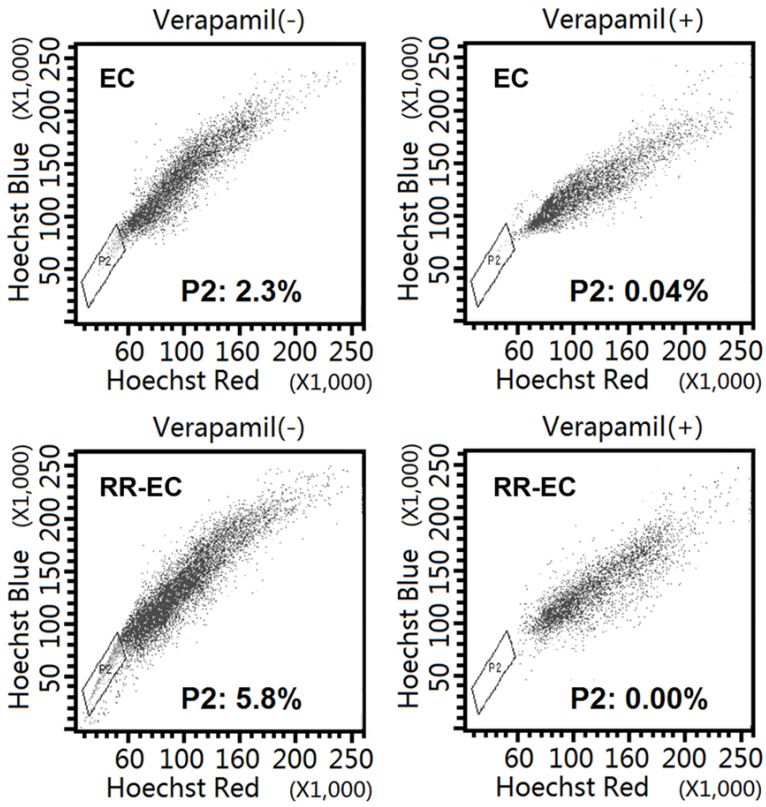

Figure 2. Flow cytometric analysis of side population (SP) in EC109 cells and RR-EC109 cells. As a control, the SP fraction was less than 0.1% within the verapamil pre-incubated cells.

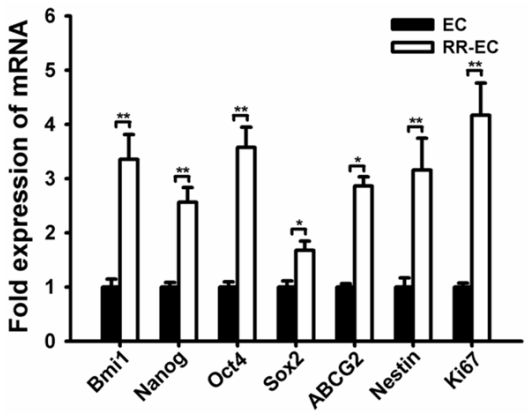

Figure 3. RR-EC109 cells express stem cell markers. *P < 0.05, **P < 0.01.

RR-EC109 cells display features of epithelial-mesenchymal transition

To further determine the function of epithelial-mesenchymal transition (EMT) in CSC production, we examined the expression of EMT markers in both RR-EC109 and EC109 cells. E-cadherin expression was lower in RR-EC109 cells than in EC109 cells, while N-cadherin, Snail, and Twist expressions were higher in RR-EC109 cells than in EC109 cells (all $P < 0.05$) (**Figure 4**).

Discussion

Cancer stem cells (CSCs) are thought to be the source of carcinogenesis, invasion, metastasis, and resistance to standard chemoradiotherapy [9, 10]. Many studies have demonstrated that the presence of CSCs in solid tumors promotes chemotherapy resistance, which explains systemic therapy failure [11-13]. Understanding the molecular process of tumor cell survival during chemotherapy may thus lead to novel therapeutic targets.

The fundamental challenge in developing a therapeutic intervention that targets esophageal squamous (ESCC) stem cells is specifically identifying and isolating CSCs. Relevant studies have not yet reported that CSCs can be identified and isolated from non-CSCs using a single approach. To detect CSCs in various malignancies, including ESCC, a combination of approaches, such as cell sorting based on cell surface and intracellular protein markers, in vitro functional tests, and xenograft is often used. However, these strategies alone cannot guarantee the total isolation of the CSC population. As a result, a combination of methodologies is employed for accurate CSC identification and isolation. In this study, RR-EC109 cells were induced by high dosage intermittent induction and increasing time over 7 months. The researchers subsequently determined whether the RR-EC109 cells were CSCs.

One of the characteristics of CSCs is their ability to generate tumor cell spheres in serum-free suspension culture, indicating the capacity for self-renewal [14]. According to the cancer stem cell theory, CSCs are cell subsets that can self-renew and differentiate into daughter cells over

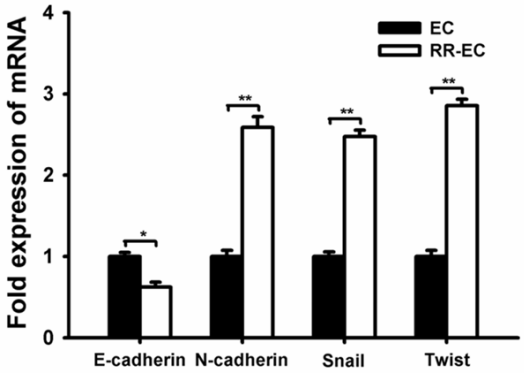

Figure 4. RR-EC109 cells display features of epithelial-mesenchymal transition. *P < 0.05, **P < 0.01.

an extended period. As a result, CSCs have a greater capacity for cloning than differentiated cells. Compared to EC109 cells, the RR-EC109 cells were shown to have a greater capacity to generate tumor spheres and a higher clonogenic efficiency. This finding implies that EC109 cells include a self-renewing population of CSCs.

It has been shown that a side population (SP) exists by flow cytometric study within many cancer cell lines with stem cell-like characteristics [15, 16]. Other research found that SP cells identified from ESCC harbor tumor-initiating cell characteristics [17]. The ATP-binding cassette (ABC) transporter is highly expressed on the membrane surface of SP cells, but the ABC transporter may pump Hoechst 33342 fluorescent dye out of the cell while displaying minimal staining. Flow cytometric examination of the RR-EC109 cells indicated a greater population of SP cells, which is consistent with the findings of other studies. However, there were essentially no lateral group cells in RR-EC109 and EC109 cells that had been pretreated with the ABC transporter inhibitor, verapamil.

A variety of biomolecules may be employed to identify and isolate CSCs in various malignancies. Although no well-defined panel of ESCC stem cell markers has been found and described, various putative molecules are being employed to functionally identify CSCs in ESCC [18]. BMI1 is a cancer-related oncoprotein that controls cell cycle events in cancer cells. Some studies have found that Bmi1 expression levels are associated with tumor regression grade and can predict early recurrence and poor prognosis in patients with ESCC following chemoradiotherapy [19, 20]. Nanog is a transcription factor that has a role in the self-renewal of embryonic stem cells. The expression of Nanog can be utilized to predict prognosis in ESCC [20].

Furthermore, Oct4 and Sox2 are transcription factors that govern stem cell stemness and development. Oct4 expression was strongly associated with high histological grade and poor survival in ESCC [21]. According to one study, ESCC cells harboring Oct4 showed a higher potential to generate tumor spheres in culture and xenotransplantation models than differentiated cells [22]. In ESCC, Sox2 protein expression has been linked to lymphatic metastasis, vascular invasion, high histologic grade, and poor survival [21, 23]. ABCG2 is an ATP-binding cassette transporter (ABC transporter) subtype that facilitates the transport of several compounds through the cell membrane. ABCG2 expression has been linked to lymph node metastases and a poor prognosis in individuals with ESCC [24]. In several studies, Nestin and Ki-67 have been linked to a poor prognosis in ESCC patients [23, 25]. Furthermore, Nestin-positive ESCC cells were involved in malignant growth and apoptosis. RR-EC109 cells also expressed increased amounts of ESCC markers (e.g., Bmi1, Nanog, Oct4, Sox2, ABCG2, Nestin, and Ki-67) in this study.

Epithelial-mesenchymal transition (EMT) is the process of transforming polarized and immotile epithelial cells into moveable mesenchymal cells. This process was first identified at various critical phases of embryonic development and has recently been connected to the migration and invasion of epithelial-derived CSCs. EMT has been shown to have a role in carcinogenesis, invasion, metastasis, recurrence, and treatment resistance [26, 27]. EMT has also been shown to induce CSCs [28]. We used quantitative PCR to explore the expression of EMT markers in paclitaxel-resistant ESCC cells to better understand their EMT features. The amount of E-cadherin in RR-EC109 cells was lower than in EC109 cells. In addition, N-cadherin, Snail, and Twist were more abundant in RR-EC109 cells. As a result, our findings are consistent with typical EMT char-

acteristics, such as the loss of epithelial markers and the growth of mesenchymal markers and transcription factors. These observations indicate that the ESCC stem cells underwent EMT.

In conclusion, paclitaxel selection enriched a subpopulation of CSCs from the esophageal cancer cell lines EC109 in this study. It was found to possess features of both CSCs and cells with EMT characteristics. We provide direct evidence that CSC-like cells exist among paclitaxel-resistant cells in ESCC and may play a role in medication resistance in ESCC. Also, this culture method can be employed as a temporary ESCC stem cell research tool.

Disclosure of conflict of interest

None.

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