·Basic Research·

Expression and significance of tumor drug resistance related proteins and β-catenin in esophageal squamous cell carcinoma

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[Abstract] Background and Objective: As chemotherapy is generally used in the clinical treatment of cancer, the problem of multidrug resistance (MDR) of tumors against the chemotherapeutic agents becomes more and more serious. It has been the major cause for the failure of the chemotherapy. We detected the expressions of β-catenin and tumor drug resistance related proteins, MRP2, P-gp, and Bcl-2, in esophageal squamous cell carcinoma (ESCC) to explore their function and correlation in the occurrence and development of MDR in ESCC. **Methods:** We used the tissue microarray technique, immunohistochemistry, and image analysis methods to detect the expressions of MRP2, P-gp, β-catenin, and Bcl-2 proteins and analyze their relationships with clinical data in a ESCC tissue microarray consisting of 582 specimens of ESCC, 294 specimens of normal mucosa, 92 specimens of basal cell hyperplasia, and 87 specimens of dysplasia adjacent to cancer tissue. **Results:** The integral optical density (IOD) of MRP2 and Bcl-2, which was $(195.7 \pm 175.9) \times 10^3$ and $(90.5 \pm 112.5) \times 10^3$, respectively, was significantly higher in ESCC than in normal mucosa, which was $(104.8 \pm 86.1) \times 10^3$ and $(25.2 \pm 46.6) \times 10^3$, respectively ($P < 0.01$). The IOD of P-gp and β-catenin, which was $(57.7 \pm 75.5) \times 10^3$ and $(32.0 \pm 47.0) \times 10^3$ respectively, was significantly lower in ESCC than in normal mucosa, which was $(114.8 \pm 106.6) \times 10^3$ and $(46.1 \pm 35.7) \times 10^3$, respectively ($P < 0.01$). According to the following order, well differentiated moderately differentiated poorly differentiated, the IOD of MRP2 increased in ESCC ($P < 0.01$). The IOD of β-catenin was higher in poorly differentiated ESCC than in well or moderately differentiated ESCC ($P < 0.01$). The IOD of Bcl-2 was lower in well differentiated ESCC than in poorly and moderately differentiated ESCC ($P < 0.01$). The IOD of β-catenin and Bcl-2 was higher in the ESCC of specimens with infiltration depths that were in membrane mucosa than those in the muscular layer or serous coat ($P < 0.01$). The IOD of Bcl-2 was significantly higher in cases with lymph node metastasis than in cases without ($P < 0.01$). Positive correlations which were respectively between the expressions of P-gp and MRP2, the expressions of P-gp and Bcl-2 were found ($r = 0.288$ and $r = 0.253$, respectively, $P < 0.01$). **Conclusions:** MRP2, P-gp, and Bcl-2 may be taken as prognostic factors for MDR of ESCC. β-catenin may play an important role in carcinogenesis and progression of ESCC.

Key words: Esophageal neoplasm, squamous cell carcinoma, MRP2, P-gp, β-catenin, Bcl-2, tissue microarray, immunohistochemistry, image analysis

Multidrug resistance (MDR) in tumor cells is the major cause for the failure of chemotherapy. There are complicated and multiple causes and mechanisms for the resistance of tumor cells to chemotherapeutics. A single tumor cell that is drug-resistant can have many mechanisms of drug resistance at the same time. The pathways of drug resistance mediated either by the ATP binding cassette (ABC) transport protein family or by the inhibition of cell apoptosis are both important and the primary focus of the present investigations of MDR. Previous studies have shown that there is a close correlation between these two factors on the MDR of tumors.[1] P-glycoprotein (P-gp) and MDR-associated protein 2 (MRP2), which are both members of the ABC transport protein family, have been found with high expressions in the epithelial tissue of the alimentary canal, including esophageal epithelial tissue.[2] Yamada *et al.*[3] found that the MDR1 gene encoding P-gp is a direct target gene of the TCF4/β-catenin transcriptional complex, which is a responsive element of the Wnt signaling pathway and the activation of the TCF4/β-catenin pathway can lead to the upregulation of the expression of P-gp. Yan *et al.*[4] found that after the activation of the TCF4/β-catenin pathway, the expressions of those genes, which are upstream

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genes and controlling genes of Bcl-2, can be upregulated in human esophageal squamous cell carcinoma (ESCC).

To explore the contributions of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC, this study applied the tissue microarray technique, immunohistochemistry, and image analysis to detect the expressions of MRP2, P-gp, β-catenin, and Bcl-2 proteins in ESCC and tissue adjacent to the cancer to investigate the correlations of them in the mechanisms involved in the occurrence and development of MDR in ESCC.

Materials and Methods

Selection of cases

A total of 582 esophageal cancer specimens were used in this study: 42 from patients at the Qingyuan Affiliated Hospital of Medical College, Jinan University, between March 1999 and January 2008; 238 from patients at Guangzhou Cancer Hospital between June 2003 and June 2007; and 302 from patients at Meizhou People Hospital between September 2004 and March 2008. All patients who received radical treatment received no radiotherapy and chemotherapy before surgery. All patients were diagnosed with ESCC independently by two pathologists. There were 387 men and 145 women, aged between 19 years and 98 years, with a median of 56 years. All tissue microarray included 582 specimens of ESCC, 294 specimens of normal mucosa, 92 specimens of basal cell hyperplasia, and 87 specimens of dysplasia adjacent to the cancer tissue.

Material and test equipment

The tissue microarrays, which consisted of 582 specimens of esophageal cancer, were made and supplied by the Department of Pathology, Medical College, Jinan University. Each specimen included four typical tissue microarray cores, the diameters of which were 1 mm (more than one was ESCC, the rest were adjacent mucosa) (Figure 1).

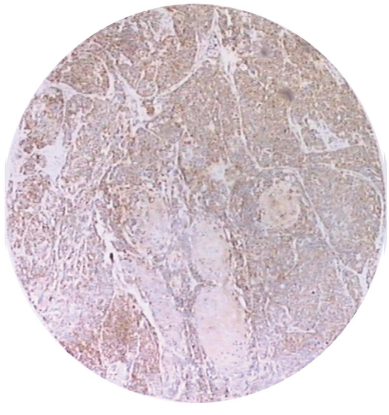

Figure 1 Tissue microarray of esophageal squamous cell carcinoma (DAB ×40)

Anti-mouse β-catenin monoclonal antibody with working solution was bought from Fuzhou Maixin Biotech. Concentrated anti-rabbit MRP2, P-gp polyclonal antibody (working dilution 1: 200 and 1:300, respectively) were brought from Beijing Boaosen Biotech. Concentrated anti-mouse Bcl-2 monoclonal antibody (working dilution 1:50) as well as the streptavidin peroxidase (SP) kit and diaminobenzidine (DAB) kits were bought from Beijing Zhongshan Biotech. Image collection was taken by a professional CCD image acquisition system (Leica Ltd, Germany) supplied by the central laboratory of Medical College, Jinan University.

Experimental process

All microarray cores were stained by SP. The procedure was carried out according to the manual of the SP kit. Microwave heating was used for antigen retrieval. Microarray cores were also treated by using the DAB kit for staining and hematoxylin for counterstaining. Phosphate buffered saline (PBS) was used in replacement of primary antibodies as the negative control, while known positive slides were used as positive controls.

Using the method proposed by the literature, the image collection was carried out by a microscopic CCD image acquisition system, and at least five high magnification fields of view (200×) were randomly selected from each core of each specimen.

Result analysis

Assessment of immunohistochemistry For MRP2 and P-gp, the areas with brown yellow granules in the cellular membrane and cytoplasm were defined as areas of positive staining (Figures 2 and 3). For β-catenin, the areas with brown yellow granules in the

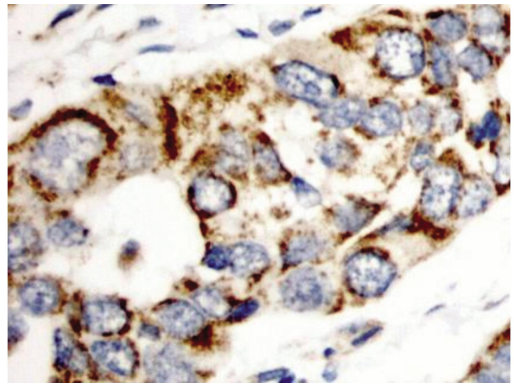

Figure 2 The expression of MRP2 in the tumor cells of esophageal squamous cell carcinoma (DAB ×200)

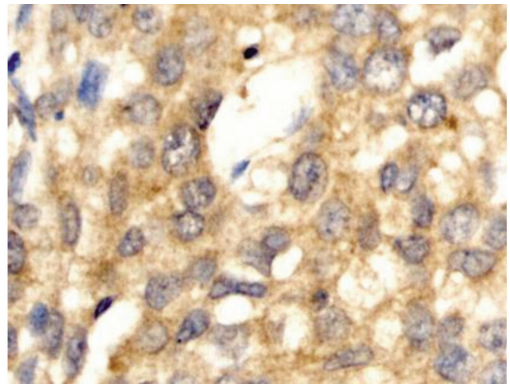

Figure 3 The expression of P-gp in the tumor cells of esophageal squamous cell carcinoma (DAB ×200)

cellular membrane, cytoplasm, and nucleus were defined as areas of positive staining (Figure 4). For Bcl-2, positive areas were defined as the areas with brown yellow granules in the cytoplasm and nucleus (Figure 5).

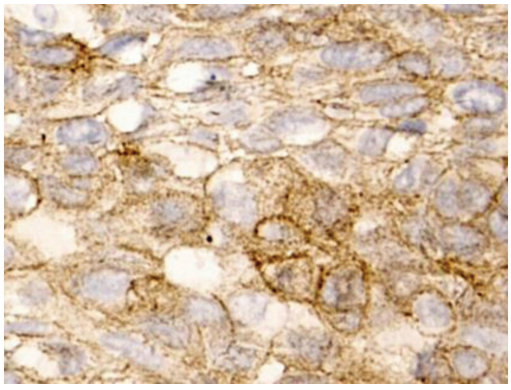

Figure 4 The expression of β-catenin in the tumor cells of esophageal squamous cell carcinoma (DAB ×200)

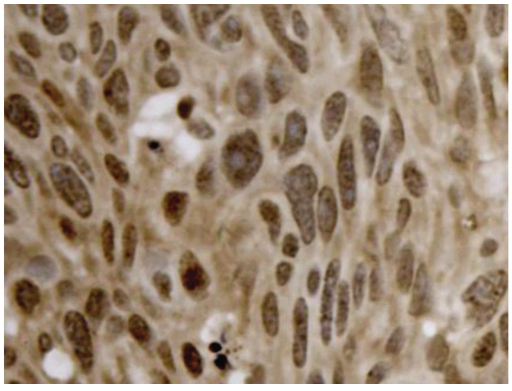

Figure 5 The expression of Bcl-2 in the tumor cells of esophageal squamous cell carcinoma (DAB ×200)

Image analysis Pictures that were taken under high magnification fields of view (200 ×) were analyzed using the professional image analysis software, Image-Pro Plus 6.0 (IPP6.0). First, we used IPP6.0 to analyze a group of pictures with positive staining areas and typical significance. According to the characteristics of positive immunohistochemical staining of each protein, we chose the definite areas of these pictures and calculated the integral optical density (IOD) in these areas. After the adjusting for operation, we confirmed and saved the installation parameters used for measurement. Then the operative procedure and installation parameters would be saved as a 'setting'. By operating this setting, we could use the photodensitometry to analyze all the pictures at the same installation parameters. We used the IOD to represent the grain density of each protein in those pictures, so it could reflect and represent the expression of each protein.

Statistical analysis

Experimental data were analyzed by the SPSS version 13.0. Differences between two groups were compared using the independent samples *t* test. The Pearson product moment correlation was used to analyze the relationships among the proteins. Variance analysis was not only used to analyze the relationships between the expressions of each protein in ESCC and tissue adjacent to the cancer and but also to analyze the relationships between the expressions of each protein in ESCC and clinical data: age, size of tumor, depth of infiltration, primary location, and so on. $P < 0.05$ indicated statistical significance.

Result

Relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2, and clinical data

The relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2, and age, sex, size of tumor, primary location, and gross appearance type are shown in Table 1.The IOD of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC had no correlation with clinical data ($P > 0.05$).

The expressions of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC and tissue adjacent to the cancer

The expressions of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC and tissue adjacent to the cancer are shown in Table 2. The IOD of MRP2 and Bcl-2 was higher in ESCC, dysplasia adjacent to the cancer, and basal cell hyperplasia than in normal mucosa ($P < 0.01$). The IOD of P-gp and β-catenin was lower in ESCC than in dysplasia adjacent to the cancer, basal cell hyperplasia, and normal mucosa ($P < 0.01$).

The relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2 and the histological differentiation of ESCC

The relationships between the expressions of MRP2, P-gp, β-catenin, and Bcl-2 and the histological differentiation of ESCC are shown in Table 3. According to the following order, well differentiation—moderate differentiation—poor differentiation, the IOD of MRP2 increased in ESCC ($P < 0.01$). The IOD of β-catenin was higher in poorly differentiated ESCC than in well and moderately differentiated ESCC ($P < 0.01$). The IOD of Bcl-2 was lower in well differentiated ESCC than in poorly and moderately differentiated ESCC ($P < 0.01$). There was no difference between the IOD of P-gp in the different differentiations of ESCC ($P > 0.05$).

The relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2 in ESCC and clinical data relevant to prognosis

The relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2 in ESCC and clinical data relevant to prognosis are shown in Table 4. The IOD of β -catenin and Bcl-2 was higher in the ESCC of specimens with infiltration depth in the membrana mucosa than in the muscular layer or serous coat ($P < 0.01$). Compared with the group negative for lymph node metastasis, the IOD of Bcl-2 in the ESCC specimens positive for lymph node metastasis was higher ($P < 0.01$). There was no difference between the IOD of MRP2, P-gp, or β-catenin in the ESCC of specimens either positive or negative for lymph node

Table 1 The relationships between the expressions of MRP2, P-gp, β-catenin, Bcl-2, and clinical data

Group	Patient No.	IOD (×10³)			
		MRP2	P-gp	β-catenin	Bcl-2
Age					
⩽ 50	169	217.5 ± 233.0	49.6 ± 59.3	34.9 ± 55.7	90.6 ± 114.0
51–60	236	198.2 ± 145.2	65.9 ± 89.2	32.3 ± 48.2	87.8 ± 112.8
61–70	132	168.0 ± 134.1	55.7 ± 69.5	28.8 ± 35.0	89.4 ± 109.3
> 70	45	181.4 ± 175.1	50.5 ± 65.2	28.3 ± 34.3	107.0 ± 117.5
Gender					
Male	437	196.4 ± 184.8	58.0 ± 73.7	32.5 ± 49.8	92.4 ± 116.0
Female	145	193.3 ± 146.1	56.8 ± 71.0	30.4 ± 37.5	84.5 ± 101.8
Primary location					
Upper esophagus	43	243.3 ± 175.7	56.9 ± 70.8	35.1 ± 43.7	94.8 ± 115.7
Median esophagus	356	191.9 ± 141.8	61.6 ± 81.2	32.2 ± 51.4	86.4 ± 106.2
Lower esophagus	183	191.9 ± 227.6	50.1 ± 64.0	30.8 ± 38.3	97.3 ± 123.5
Size of tumor(cm)					
⩽ 3	239	206.4 ± 219.5	54.4 ± 81.0	35.0 ± 59.2	93.8 ± 120.1
3–5	164	196.1 ± 146.3	57.7 ± 70.4	30.2 ± 36.7	86.3 ± 104.8
> 5	179	181.0 ± 128.7	62.0 ± 72.5	29.6 ± 35.6	89.8 ± 109.4
Gross appearance					
Ulcer type	410	191.3 ± 184.4	58.5 ± 78.2	32.4 ± 50.3	89.2 ± 111.5
Medullary type	56	180.0 ± 138.3	62.7 ± 76.8	30.1 ± 35.9	99.7 ± 122.1
Sclerotic type	37	197.2 ± 123.6	59.2 ± 73.4	34.8 ± 43.5	100.4 ± 126.5
Mushroom type	79	228.9 ± 173.7	49.3 ± 60.6	29.7 ± 37.1	85.6 ± 106.0

IOD: integral optical density.

Table 2 The expressions of MRP2, P-gp, β-catenin, Bcl-2 in esophageal squamous cell carcinoma and tissue adjacent to the cancer

Group	Patient No.	IOD (×10³)			
		MRP2	P-gp	β-catenin	Bcl-2
ESCC	582	195.7± 175.9	57.7 ± 75.5**	32.0 ± 47.0**	90.5 ± 112.5
Dysplasia	87	227.8 ± 149.7	150.9 ± 116.1	56.4 ± 47.0	81.4 ± 114.7
Basal cell hyperplasia	92	163.4 ± 102.6	119.4 ± 119.5	70.0 ± 60.6	59.7 ± 74.0
Normal mucosa	294	104.8 ± 86.1*	114.8 ± 106.6	46.1 ± 35.7	25.2 ± 46.6*

ESCC, esophageal squamous cell carcinoma. *P < 0.01, vs. ESCC or dysplasia adjacent or basal cell hyperplasia; **P < 0.01, vs. dysplasia adjacent or basal cell hyperplasia or normal mucosa.

Table 3 The expressions of MRP2, P-gp, β-catenin, and Bcl-2 in esophageal squamous cell carcinoma of different histological differentiations

Differentiation	Patient No.	IOD (×10³)			
		MRP2	P-gp	β-catenin	Bcl-2
Well	139	146 ± 101.4*	59.6 ± 79.1	22.0 ± 28.9	55.0 ± 76.9*
Moderate	363	196.7 ± 189.7	58.4 ± 78.6	30.0 ± 51.1	100.6 ± 124.8
Poor	80	277.5 ± 182.9**	51.1 ± 51.3	58.5 ± 43.7**	106.1 ± 92.4

*P < 0.01, vs. moderate or poor differentiation; **P < 0.01, vs. well or moderate differentiation.

metastasis (P > 0.05). The IOD of MRP2 and P-gp in the ESCC of specimens with different infiltration depths of ESCC had no difference with each other (P > 0.05).

The correlations of the expressions of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC

Positive correlations were found between the IOD of P-gp and the IOD of MRP2 and Bcl-2 (r = 0.288, P < 0.001; r = 0.253, P = 0.004, respectively). There was no correlation between the IOD of other proteins in ESCC (P > 0.05).

Discussion

By using immunohistochemistry and image analysis, we took the IOD of each protein as reference data to detect and analyze their expressions in ESCC and in tissue adjacent to the cancer. In our study, the expression of β-catenin was lower in ESCC than in adjacent dysplasia, basal cell hyperplasia, and normal mucosa, which was not consistent with recent studies[5-7]. Previous studies

Table 4 The relationships between the expressions of MRP2, P-gp, β-catenin, and Bcl-2 in ESCC and clinical data relevant to prognosis

Group	Patient No.	IOD ($\times 10^3$)			
		MRP2	P-gp	β-catenin	Bcl-2
Infiltration depth					
Mucosa	47	241.2 ± 158.8	40.6 ± 59.4	63.3 ± 43.9*	139.9 ± 115.3*
Muscular layer	160	195.1 ± 137.7	50.2 ± 60.3	28.7 ± 43.3	84.0 ± 100.2
Serous coat	375	190.2 ± 191.3	63.0 ± 82.4	29.5 ± 47.6	87.0 ± 116.0
Lymph node metastasis					
Positive	191	215.5 ± 225.2	54.6 ± 69.5	35.8 ± 54.8	109.5 ± 126.7**
Negative	391	186.0 ± 145.2	59.1 ± 78.3	30.1 ± 42.7	81.1 ± 103.9

*$P < 0.01$, vs. muscular layer or serous coat; **$P < 0.01$, vs. lymph node metastasis negative.

have usually been based on two study methods. The first one is that by using the study of cytobiology, the change of the expression of β-catenin in single cell can be detected.[5] By this method, it can reflect changes in the expression of β-catenin inside the cells. But this change cannot be contrasted and referenced with clinical data, so it lacks clinical relevance. The second one is that by immunohistochemistry, the positive rate of the expression of β-catenin in ESCC can be taken as a reference index.[6,7] However there are major issues of relative accuracy and subjectivity by this method. By using tissue microarray, multi-tissues can be fixed on the sustentaculum. So it can detect and analyze (high-flux and multi-sample) many samples at the same time, under controlled experimental conditions, and eliminate the relative accuracy between different batches of an experiment. In addition, it can cut down on the reagent dosage and greatly raise the efficiency of detection. In this study, by using tissue microarray and taking the IOD as the index, we used semiquantitative analysis to study the expression of β-catenin in ESCC and the tissue adjacent to the cancer to obtain more objective and true conclusions.

β-catenin is the key signaling component of Wnt signaling pathway. As the Wnt pathway is not activated, most β-catenin inside cells bind to E-cadherin and α-catenin on the cell membrane to form a complex epidermal catenin and cadherin unit (ECCU). The rest is phosphorylated after binding to a protein degradation complex in the cytoplasm, consisting of molecules, such as glycogen synthase kinase (GSK-3β) and adenomatous polyposis coli (APC), and then finally degraded and polyubiquitinated. After a number of cytokines bind to receptors on the cellular membrane, β-catenin dissociates with E-cadherin and α-catenin on the cellular membrane and gets into the cytoplasm. The degradation complex consisting of β-catenin, GSK-3β , and APC fails to degrade β-catenin and β-catenin accumulates in the cytoplasm without degradation, and translocates to the nucleus, where it binds to Tcf/Lef (T cell factor/ lymphoid enhancer factor) and initiates the transcription of a number of target genes of the Wnt pathway, which then leads to carcinogenesis. The accumulation of β-catenin in the cytoplasm and nucleus indicates the activation of the Wnt pathway. One study showed that the accumulation of β-catenin in the cytoplasm and nucleus not only needs that the degradation complex consisting of β-catenin, GSK-3β, and APC fails to degrade β-catenin, but also needs the stabilization of β-catenin in the cytoplasm and is still influenced by a number of factors, including presenilin1 (PS1) and so on, which can promote the degradation of β-catenin[8]. Previous studies have shown that as the expression of β-catenin increases, the Wnt pathway can be activated, leading to carcinogenesis[8]. In this study, the expression of β-catenin is lower in ESCC than in tissue adjacent to the cancer. Although some β-catenin accumulates in the cytoplasm and nucleus, after β-catenin dissociates from the cellular membrane, there still may be too much β-catenin degraded in ESCC. To be confirmed. this inference needs further research. We showed that the expression of β-catenin was higher in poorly differentiated ESCC than in well and moderately differentiated ESCC, which is similar to the findings of other studies.[6,7] With the accumulation of β-catenin, the Wnt pathway was activated and the abnormal expression of β-catenin was negatively correlated with ESCC differentiation. These data indicate that the Wnt signaling pathway plays an important role in the differentiation of ESCC. The expression of β-catenin is higher in the ESCC of specimens with infiltration depth in membrana mucosa than in the muscular layer or serous coat. This result suggests that β-catenin plays an important role in the early infiltration of ESCC. Therefore, our data implies that β-catenin plays an important role in the occurrence and development of ESCC.

As important members of the ATP binding cassette (ABC) transport protein family, P-gp and MRP2 are types of proteins with an energy-dependent efflux pump. Their expressions are found in many kinds of solid tumors and normal tissue. Moreover they play important roles in maintaining many important physiologic functions of the human body.[2] The expression of P-gp is lower in ESCC than in tissue adjacent to the cancer, but there was no difference among its expression in dysplasia adjacent to the cancer, basal cell hyperplasia, or normal mucosa. This result is consistent with related studies.[9] The expression of MRP2 is higher in dysplasia adjacent to the cancer, basal cell hyperplasia, and normal mucosa than in ESCC. But there is also no difference between its expression in tissue adjacent to the cancer (dysplasia adjacent, basal cell hyperplasia, or normal mucosa). This implies that in the early stages of the occurrence and development of ESCC, as the expression of MRP2 increases, MRP2 may play a more and more important role on

MDR of ESCC, which may increase too. According to the following order well differentiation—moderate differentiation–poor differentiation—the expression of MRP2 increases in ESCC. This result indicates that MRP2 may play a more important role on MDR of ESCC with poorer differentiation than on MDR of ESCC with better differentiation. Positive correlation was found between the expressions of MRP2 and P-gp, which implies that P-gp and MRP2 may have a synergistic effect on MDR of ESCC. P-gp and Bcl-2 are important members of two different MDR mechanisms. Recent studies found positive correlations between the expressions of P-gp and Bcl-2[10]. Hong *et al.*[11] found that as the expression of zinc ribbon domain-containing 1 (ZNRD1) was upregulated, it promoted the expressions of P-gp and Bcl-2 and increased MDR of tumor cells. It inferred that P-gp and Bcl-2 may have a synergistic effect on MDR in vitro. In our study, we found that there were positive correlations between the expression of P-gp and the expressions of MRP2 and Bcl-2. These data suggest that P-gp may have a synergistic effect with MRP2 and Bcl-2 and all of them may be used as prognostic factors for MDR of ESCC.

Hong *et al.*[3] found that as β -catenin accumulates inside cells, both the TCF4/β-catenin pathway can be activated and the expression of P-gp can be upregulated in vitro. However in our study, no correlations were found between the expression of β-catenin and expressions of P-gp, Bcl-2, or MRP2. The mechanism of MDR of tumors is a complicated process, which is influenced by many factors, mechanisms, pathways, and genes. To identify the relationships between drug resistance factors, we need further research.

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