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RESEARCH

Multidrug Resistance in Primary Tumors and Metastases in Patients with Esophageal Squamous Cell Carcinoma

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Abstract Studies have demonstrated that radical esophagectomy can significantly prolong disease-free survival and improve the survival rate of patients with T3 or T4 esophageal cancer and lymph node metastasis. Multidrug resistant cancer cells have active efflux mechanisms that prevent the accumulation of chemotherapeutic drugs in the cells. The purpose of this study was to compare the expression of five MDR related proteins between primary tumors in patients with thoracic esophageal squamous cell carcinoma (ESCC) and metastatic cancer in lymph nodes to explore the clinical significance of heterogeneity in MDR metastatic cancer cells. Fifty-four patients with ESCC and lymph node metastasis were included. All patients underwent subtotal esophagectomy and D2/D3 lymph node resection. The expression of lung resistance-related protein (LRP), P-glycoprotein, topoisomerase-II, thymidylate synthase, and glutathione S-transferase P1–1 (GST-π) were determined in the primary tumors and lymph nodes via immunohistochemistry. The expression of LRP was significantly different between the primary tumors and lymph nodes (P=0.026). No significant differences were found for the other four proteins, and protein expression was not associated with either degree of differentiation or disease stage. It was also found that GST-π was expressed in all patients in both the primary tumors and lymph nodes, suggesting that the design and application of chemotherapeutic protocols capable of reducing GST-π expression may be beneficial for patients with ESCC. Additional research regarding the clinical utility of MDR protein expression in ESCC is warranted to design effective chemotherapeutic protocols.

Keywords Esophageal cancer · Lymph node metastasis · Multidrug resistance · Protein expression

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Introduction

Esophageal cancer is associated with a very poor prognosis, with overall survival estimated to be a mere 14–30 % [1]. The poor prognosis is partly due to the fact that most patients are already at an advanced stage of disease at the time of diagnosis [1–4]. Studies have demonstrated that radical esophagectomy can significantly prolong disease-free survival (DFS) and improve the survival rate of patients with T3 or T4 esophageal cancer and lymph node metatastasis [3–6]. Surgery remains the mainstay of treatment for esophageal cancer; however, adjuvant chemotherapy and radiotherapy are important components of a multidisciplinary approach to treatment [1, 7–9]. Postoperative chemotherapy targets the metastatic cancer in the remaining lymph nodes, but the heterogeneity in multidrug resistance (MDR) of metastatic cancer cells is a main factor affecting chemotherapeutic efficacy [10–12].

MDR cancer cells have active efflux mechanisms that prevent the accumulation of chemotherapeutic drugs in cells [10, 12]. Adenosine triphosphate binding cassette (ABC) transporters provide protection against endogenous molecules; however, they also cause the active efflux of chemotherapeutic drugs from cells [12]. Alternatively, MDR can be conferred to cancer cells by changes in the expression of various enzymes that are either primary targets of a chemotherapeutic agent or play a role in converting a drug into an appropriate transporter substrate [13]. A number of MDR proteins have been identified to date. P-glycoprotein (P-gp), for example, confers resistance to a number of cationic and neutral chemotherapeutic agents (e.g., paclitaxel, etoposide,

vinblastine, doxorubicin) and can be inhibited by verapamil and cyclosporine [12]. High expression of glutathione S-transferase P1-1 (GST-π) reportedly correlates with resistance to alkylating agents and platinum-containing agents (e.g., cisplatin) [13]. Lung resistance-related protein (LRP) confers resistance to alkylating agents, platinum-containing agents, and alkaloids [13, 14], and cells expressing high levels of thymidylate synthase (TS) are resistant to 5-fluorouracil [15]. Topoisomerase II (TOPO-II) is not involved in the efflux of antineoplastic agents, but is a primary target for anthracycline agents, etoposide, and ellipticine. As a result, cells with low or negative expression of TOPO-II are resistant to those three agents [16]. Numerous studies have also shown that all of the above-mentioned proteins are associated with the MDR phenotype in various cancers [10, 11, 17].

Some recent publications have focused on MDR in esophageal squamous cell carcinoma (ESCC) but those studies only examined the primary lesion [11, 17]. It is known, however, that heterogeneity can exist between malignant cells in the primary tumor and metastases [18]. The purpose of this study was to compare the expression of five MDR related proteins (LRP, P-gp, TOPO-II, TS, and GST-π) between the primary lesion in patients with thoracic (TESCC) and metastatic cancer in lymph nodes to explore the clinical significance of heterogeneity in MDR metastatic cancer cells.

Materials and Methods

Patients

Patients with pathologically confirmed TESCC and metastatic cancer in≥ 1 lymph nodes were prospectively enrolled into this study. Patients with distant metastases (e.g., liver, lung) were excluded. All included patients underwent subtotal esophagectomy and D2/D3 lymph node resection in the Cancer Hospital of Sichuan Province in Chengdu, China. Chemotherapy and radiotherapy were not performed prior to surgery.

Cancer stage was determined during the postoperative pathologic examination, and included primary tumor invasion (T), the status of regional lymph nodes (N), and the status of distant metastases (M) according to the AJCC Cancer Staging Manual, 5th ed [19]. This study was approved by the Institutional review board of the hospital, and all patients provided written informed consent.

Immunohistochemistry

Both the excised primary lesions and metastatic lymph nodes were fixed in 10 % neutral formalin then dehydrated with a SakuraVIP-E300F automatic tissue dehydration unit. Specimens were cut into 4 μm sections with a Lica 2245 microtome.

Immunohistochemistry was performed with a labeled dextran polymer (LDP) method according to manufacturer's instructions and as describe previously (EnVision; Dako, Carpinteria, CA, USA) [20, 21]. In brief, the LDP method is a two-step immunohistochemical staining technique based on a peroxidase-labeled dextran polymer that is conjugated with secondary antibodies. Deparaffinized sections were treated with 3 % hydrogen peroxide for 5 min at room temperature to block endogenous peroxidase activity. The slides were rinsed with phosphate buffered saline before they were incubated with antigen retrieval solution in a microwave for 20 min. The slides were then incubated with mouse monoclonal primary antibodies against GST- π, LRP, P-gp, TOPO-II, or TS (all from Neomarker, Fremont, CA, USA) at a dilution of 1:100 for 30 min at room temperature. After rinsing with phosphate buffered saline, the slides were incubated with the secondary antibody (Dako, Carpinteria, California, USA; 1:100 dilution) for 30 min at room temperature. The slides were again rinsed in phosphate buffered saline and peroxidase was revealed by immersion in 3,3-diaminobenzidine according to the manufacturer's instructions. After washing in a stream of water for 10 min, the sections were counterstained with hematoxylin and mounted for microscopic examination. Both positive controls (positive archived tissue sections) and negative controls (phosphate buffered saline) were analyzed.

The expression of GST-π- and LRP (which are primarily found in the cytoplasm), TS (which is primarily found in the nucleus), and P-gp (in the cell membrane) were assessed. Positive was defined as the presence of homogeneous, granule-like or linear brown-yellow signals in either the cytoplasm or cell membrane. Sections were considered positive for TOPO-II if yellow or brown signals in nucleus were noted. Ten random fields at a magnification of 400× were evaluated, and the proportion of positive cells was calculated using the following equation:

$$\text{Proportion of positive cells} = (\text{total number of positive cells}/\text{total number of cells}) \times 100.$$

Results were classified as no expression (score of 0) in cases with no positive cancer cells and no brown-yellow signals; low expression (score of 1) for cases in which the proportion of positive cells was<25 % and brown-yellow signals were weak; moderate expression (score of 2) for cases in which the proportion of positive cells was 25–50 % and brown-yellow signals were strong; and high expression (score of 3) for cases in which the proportion

Table 1 Patient demographics and clinical characteristics in 54 patients diagnosed with esophageal squamous cell carcinoma

Characteristic	Value
Age (years)	58.96±7.58 (range, 42 to 75)
Sex (male/female)	45/9
Differentiation	
Poor	25 (46.3 %)
Moderate	16 (29.6 %)
Good	13 (24.1 %)
Stage	
IIB	7 (13 %)
III	41 (75.9 %)
IVA	1 (1.8 %)
IVB	5 (9.3 %)
T stage	
1	1 (1.8 %)
2	8 (14.9 %)
3	38 (70.3 %)
4	7 (13 %)
N stage	
1	54 (100 %)
0	0 (0 %)
M stage	
0	48 (88.9 %)
1a	1 (1.8 %)
1b	5 (9.3 %)

Continuous data were summarized as mean ± standard deviation and with range. Categorical data were presented as number (%)

of positive cells was >50 % and brown-yellow signals were extremely strong. Each section was independently by two pathologists who were blinded to the origin of the tissue. The overall reported result from each pathologic section was the average of the two pathologists' independent interpretations.

Determination of MDR

Overexpression of LRP, P-gp, TS, or GST-π is associated with the MDR phenotype [12–15]. Thus, moderate to high expression of LRP, P-gp, TS, or GST-π indicates resistance to the corresponding chemotherapeutics, and no or low expression indicates sensitivity to the corresponding chemotherapeutics. Underexpression of TOPO-II is associated with the MDR phenotype [16]. Thus, low or no expression of TOPO-II indicates resistance to chemotherapeutics, and moderate to high expression indicates sensitivity to chemotherapeutics.

Statistical Analysis

Patient demographics and clinical characteristics were summarized as mean ± standard deviation (SD), range was used for age and survival times, and n (%) for categorical data. Protein expression levels were expressed as n (%) for both the lymph nodes and tumors and levels were compared using the Wilcoxon signed-rank test (because the expression levels were ordinal data). Differences between differentiation levels or tumor stages were compared using the Kruskal Wallis test. All statistical analyses were two-tailed, and a $P< 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 18.0 statistics software (SPSS Inc, Chicago, IL, USA).

Results

As described in Tables 1 and 2, 54 patients (45 males) were enrolled in this study. In total, 46.3 % (25/54) tumors were poorly differentiated, 29.6 % (16/54) were moderately differentiated, and 24.1 % (13/54) were well differentiated. Among the 54 patients, 41 (75.9 %) had stage III disease, 7 (13 %) had stage IIB, 5 (9.3 %) had stage IVB, and 1 (1.8 %) had stage IVA. In total, there were seven positive

Table 2 Comparison of the MDR protein expression level between primary ESCC tumors and metastasized lymph nodes

		Protein expression level				
Protein	Location	0 (none)	1 (low)	2 (moderate)	3 (high)	*P* value
GST-π	Tumor	0 (0 %)	1 (1.9 %)	9 (16.6 %)	44 (81.5 %)	1.000
	Lymph node	0 (0 %)	2 (3.7 %)	7 (13 %)	45 (83.3 %)	
LRP	Tumor	18 (33.3 %)	19 (35.2 %)	13 (24.1 %)	4 (7.4 %)	0.026
	Lymph node	31 (57.4 %)	12 (22.2 %)	7 (13.0 %)	4 (7.4 %)	
P-gp	Tumor	22 (40.7 %)	21 (38.9 %)	9 (16.7 %)	2 (3.7 %)	0.923
	Lymph node	22 (40.7 %)	23 (42.6 %)	7 (13.0 %)	2 (3.7 %)	
TOPO-II	Tumor	19 (35.2 %)	22 (40.7 %)	12 (22.2 %)	1 (1.9 %)	0.435
	Lymph node	26 (48.1 %)	13 (24.1 %)	14 (25.9 %)	1 (1.9 %)	
TS	Tumor	20 (37.0 %)	23 (42.6 %)	8 (14.8 %)	3 (5.6 %)	0.646
	Lymph node	22 (40.7 %)	23 (42.6 %)	5 (9.3 %)	4 (7.4 %)	

Data were expressed as number (%)

lymph nodes under the carina, seven adjacent to the left recurrent laryngeal nerve, eight adjacent to right recurrent laryngeal nerve, five adjacent to the primary tumor, two in the thoracic duct, six adjacent to the cardia of stomach, 11 adjacent to the left gastric vessels, seven in the neck, one adjacent to the arch of the azygos vein, and two adjacent to the lower esophagus.

Figure 1 shows representative photomicrographs the expression of GST-π, LRP, P-gp, TOPO-II, and TS in ESCC primary tumors, and Fig. 2 shows representative photomicrographs of the expression of the five MDR proteins in metastasized lymph nodes. Only the difference in LRP expression was significantly different between the primary tumors and the lymph nodes (P=0.026). Among the five MDR proteins, only GST-π was expressed in both the primary (tumor) and secondary (lymph node) cancer sites in all patients. LPR was not expressed in the tumors of 18 patients and was absent in the lymph nodes of 31 patients. P-gp was not expressed in the tumors of 22 patients and was not found in the lymph nodes of 22 patients. TOPO-II was absent in the tumors of 19 individuals and was not found in the lymph nodes of 26 patients. The tumors of 20 patients did not express TS, and lymph nodes from 22 patients were negative for TS. Among all 54 patients, 15 patients were

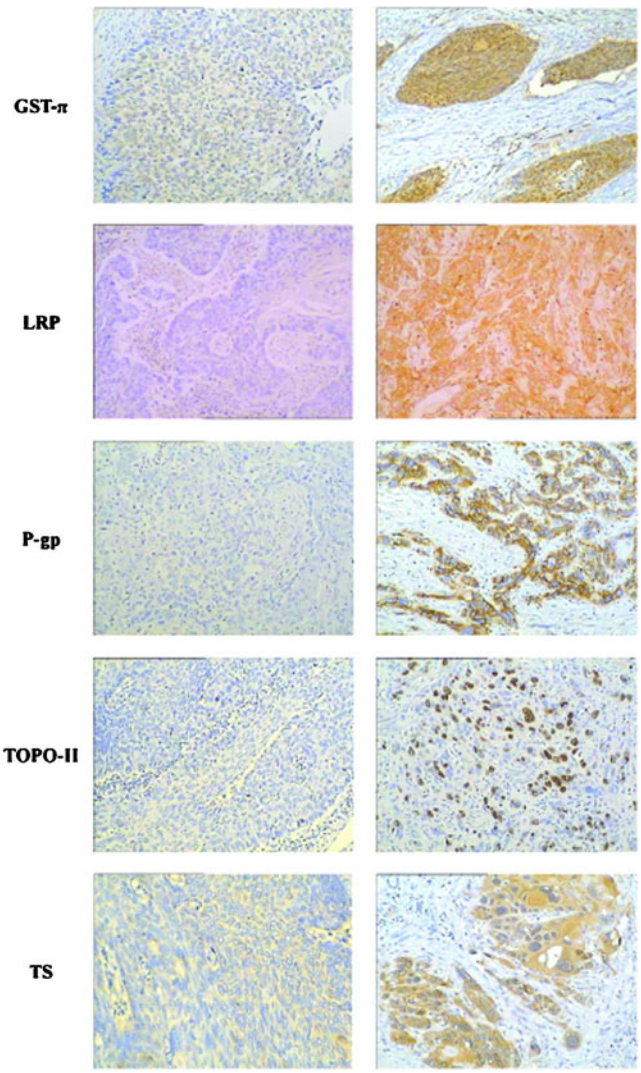

Fig. 1 Examples of the expression of multidrug-resistant (MDR) proteins in primary tumors in patients diagnosed with esophageal squamous cell carcinoma (ESCC). A: Weak positive expression of GST-π (+; left) and strong positive expression (+++; right). B: Negative expression of LRP (-; left) and strong positive expression (+++; right). C: Negatvie expression of P-gp (-; left) and strong positive expression (+++; right). D: Negative exprssion of TOPO-II (-; left) and positive expression (++; right). E: Negative expression of TS (-; left) and strong positive expression (+++; right). Original magnification × 100

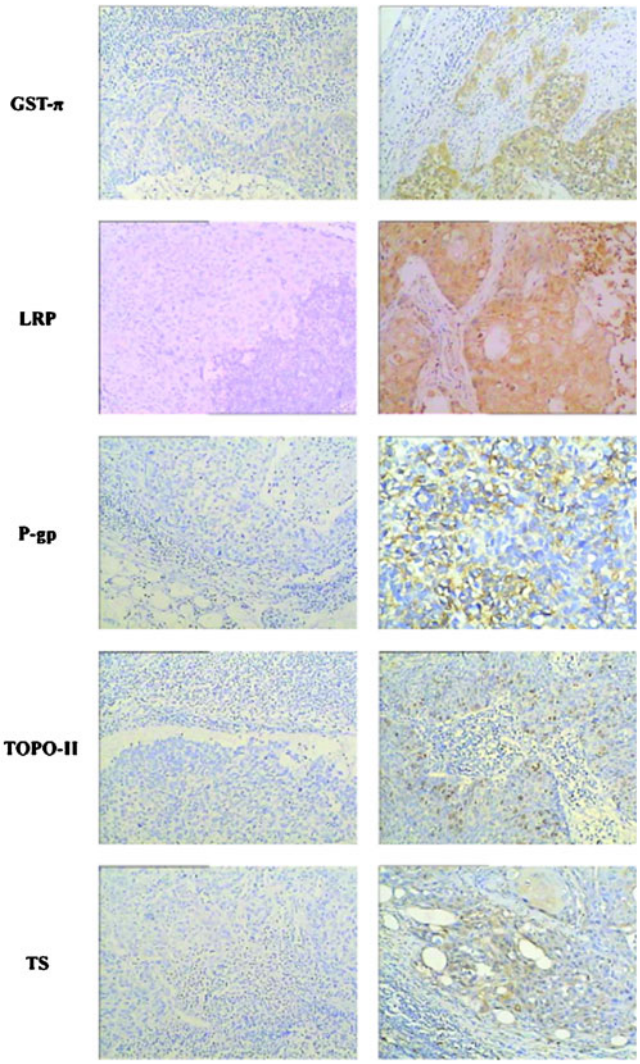

Fig. 2 Examples of the expression of MDR proteins in patients diagnosed with ESCC and with metastases to lymph nodes. A: Weak positive expression of GST-π (+; left) and strong positive expression (+++; right). B: Negative expression of LRP (-; left) and strong positive expression (+++; right). C: Negative expression of P-gp (-; left) and positive expression (++; right). D: Negative exprssion of TOPO-II (-; left) and positive expression (++; right). E: Negative expression of TS (-; left) and positive expression (++; right). Original magnification × 100

negative for LRP expression in both the primary tumor and lymph nodes, 15 patients were negative for P-gp expression in both the primary tumor and lymph nodes, TOPO-II was absent from both the primary tumor and lymph nodes in 16 patients, and there was no expression of TS in either the primary tumors or lymph nodes in 12 patients.

The protein expression between tumors and lymph nodes in the same patient were classified into three categories: upregulated (if the expression of the particular protein was higher in the metastatic lymph nodes than in the primary tumor); consistent (if both the primary and metastatic sites had the same level of protein expression); and downregulated (if the expression of the MDR protein was lower in the metastatic lymph nodes than in the primary tumor). None of the protein expression profiles were significantly associated with the either degree of differentiation (Table 3), disease stage (Table 4), overall survival (OS), or DFS (Table 5).

Discussion

Few studies have examined differences in MDR protein expression between primary lesions and lymph nodes in ESCC. The results of this study showed that although there were differences in the expression of the five different examined proteins between the two different tumor types, there was little difference in the expression of the proteins between the primary lesion and metastasized lymph nodes in the same patient with the exception of LRP.

Esophageal cancer is usually already at a middle or late stage when patients undergo radical esophagectomy. In addition, the incidence of lymph node metastasis is extremely high, which is the main factor affecting prognosis [22, 23]. Chemotherapy following surgical intervention can prolong survival times in esophageal cancer patients with lymph node metastasis, but the OS rate does not generally appear to increase [3, 5]. One study conducted in the Shanghai Cancer Hospital, however, showed that postoperative chemotherapy could increase the survival rate of esophageal cancer patients with carotid and abdominal lymph node metastasis [24], and there is evidence that perioperative chemotherapy is an independent predictor of long-term survival following esophagectomy [2]. Although postoperative chemotherapy is critical for improving the survival of esophageal cancer patients, it fails to control the postoperative lymph node metastasis in a small number of patients [3, 6].

Resistance of ESCC to chemotherapeutic agents is not uncommon, and studies are beginning to show that genetic factors can influence outcomes. For example, Wang et al. [25] found that an inverse expression of dihydrodiol dehydrogenase and GST-π was associated with survival in patients ESCC.

Table 3 Associations of MDR protein expression in primary tumors and lymph nodes based on degree of differentiation

Protein	Differentiation			*P* value
	Poor (n=25)	Moderate (n=16)	Well (n=13)	
GST-π				0.846
Upregulated	3 (12.0 %)	1 (6.3 %)	0 (0 %)	
Consistent	19 (76.0 %)	14 (87.4 %)	12 (92.3 %)	
Downregulated	3 (12.0 %)	1 (6.3 %)	1 (7.7 %)	
LRP				0.769
Upregulated	10 (40 %)	6 (37.5 %)	5 (38.5 %)	
Consistent	10 (40 %)	10 (62.5 %)	5 (38.5 %)	
Downregulated	5 (20 %)	0 (0 %)	3 (23.0 %)	
P-gp				0.549
Upregulated	5 (20 %)	3 (18.8 %)	3 (23.1 %)	
Consistent	13 (52.0 %)	9 (56.3 %)	9 (69.2 %)	
Downregulated	7 (28.0 %)	4 (25.0 %)	1 (7.7 %)	
TOPO-II				0.131
Upregulated	9 (36 %)	7 (43.8 %)	0 (0 %)	
Consistent	11 (44 %)	6 (37.5 %)	10 (76.9 %)	
Downregulated	5 (20 %)	3 (18.8 %)	3 (23.1 %)	
TS				0.237
Upregulated	6 (24 %)	7 (43.8 %)	0 (0 %)	
Consistent	12 (46 %)	6 (37.5 %)	11 (84.6 %)	
Downregulated	7 (28 %)	3 (18.8 %)	2 (15.4 %)	

Data were expressed as number (%)

Table 4 Associations of MDR protein expression in primary tumors and lymph nodes based on disease stage

Proteins	Stage				*P* value
	IIB (*n*=7)	III (*n*=41)	IVA (*n*=1)	IVB (*n*=5)	
GST-π					0.320
Upregulated	1 (14.2 %)	3 (7.3 %)	0 (0 %)	0 (0 %)	
Consistent	3 (42.9 %)	36 (87.8 %)	1 (100 %)	5 (100 %)	
Downregulated	3 (42.9 %)	2 (4.9 %)	0 (0 %)	0 (0 %)	
LRP					0.813
Upregulated	2 (28.6 %)	17 (41.5 %)	0 (0 %)	2 (40 %)	
Consistent	4 (57.1 %)	19 (46.3 %)	1 (100 %)	1 (20 %)	
Downregulated	1 (14.3 %)	5 (12.2 %)	0 (0 %)	2 (40 %)	
P-gp					0.888
Upregulated	1 (14.3 %)	8 (19.5 %)	0 (0 %)	2 (40 %)	
Consistent	5 (71.4 %)	23 (56.1 %)	1 (100 %)	2 (40 %)	
Downregulated	1 (14.3 %)	10 (24.4 %)	0 (0 %)	1 (20 %)	
TOPO-II					0.465
Upregulated	4 (57.1 %)	11 (26.8 %)	0 (0 %)	1 (20 %)	
Consistent	2 (28.6 %)	22 (53.7 %)	1 (100 %)	2 (40 %)	
Downregulated	1 (14.3 %)	8 (19.5 %)	0 (0 %)	2 (40 %)	
TS					0.868
Upregulated	2 (28.6 %)	10 (24.4 %)	0 (0 %)	1 (20 %)	
Consistent	4 (57.1 %)	20 (48.8 %)	1 (100 %)	4 (80 %)	
Downregulated	1 (14.3 %)	11 (26.8 %)	0 (05)	0 (0 %)	

Data were expressed as number (%)

Table 5 Association of overall survival and disease-free survival and MDR protein expression level

Protein expression	OS	P value	DFS	*P* value
GST-π		0.215		0.310
Upregulated	21.5±7.05 (13,30)		18.75±9.43 (10,30)	
Consistent	23±10.26 (3,41)		20.53±10.47 (2,41)	
Downregulated	31.4±15.14 (21,58)		31.4±15.14 (21,58)	
LRP		0.274		0.627
Upregulated	25.52±10.4 (3,41)		23.67±10.73 (2,41)	
Consistent	20.56±7.33 (6,36)		17.96±7.73 (5,30)	
Downregulated	28.5±17.25 (8,58)		26.25±17.87 (6,58)	
P-gp		0.012*		0.666
Upregulated	28.18±11.64 (8,41)		25.91±12.19 (6,41)	
Consistent	23.97±10.08 (3,58)		21.74±10.47 (2,58)	
Downregulated	18.75±10.06 (6,36)		16.42±10.72 (5,35)	
TOPO-II		0.841		0.538
Upregulated	23.06±9.57 (6,36)		21.31±9.92 (5,34)	
Consistent	24±11.41 (3,58)		21.22±11.81 (2,58)	
Downregulated	23.73±11.27 (9,41)		22±12.07 (7,41)	
TS		0.583		0.724
Upregulated	22.69±9.3 (8,36)		20.23±9.71 (6,33)	
Consistent	22.83±11.51 (3,58)		20.48±12.3 (2,58)	
Downregulated	26.75±10.18 (6,39)		24.92±9.58 (5,36)	

OS time and DFS were summarized as mean ± standard deviation (range), and differences between MDR protein expression levels were compared using the Log-rank test

DFS disease-free survival; *OS* overall survival; *MDR* multidrug-resistant

Resistance to chemotherapeutics, either natural or acquired, is a major challenge in the treatment of cancer [10]. Malignancies are usually a result of monoclonal proliferation of malignantly transformed cells; however, changes in genes or macromolecules can lead to the development of subcolonies with individual characteristics and heterogeneity in their sensitivity to chemotherapeutics [10]. Resistance has been noted against almost every chemotherapeutic agent, and various mechanisms have been identified that can act either individually or synergistically within the same lesion, resulting in MDR [10].

Interestingly, studies are showing that identification of ABC transporters may result in assays that can predict an individual's response to different chemotherapeutic agents [26, 27]. For example, one study by Shi et al. [28] found marked variation in the expression of GST-π, P-gp, TOPO-II, and LRP in primary gastric adenocarcinoma and correlations with metastases, differentiation, and clinicopathologic staging. Funke et al. [29] reported that genetic polymorphisms in GST genes were associated with survival of colorectal cancer patients treated chemotherapeutically. A molecular study by Becker et al. [18] of primary breast cancer and metastatic breast cancer in axillary lymph nodes showed that metastatic tumors showed a different pattern of chromosomal changes than the primary lesion.

In the current study, only a difference in LRP expression was noted between lymph nodes and tumors. Specifically, LRP expression was higher in the primary tumor than the metastatic lymph nodes. This finding could be an important consideration for the post-operative selection of an appropriate chemotherapy regimen that should target the metastatic lymph nodes. For example, platinum containing drugs and plant alkaloids could be considered that would theoretically reduce LRP expression in the nodes. Further research to support this hypothesis is needed, however, prior to making this a widespread clinical recommendation.

Although the results of the current study did not indicate differences between the expression of the remaining four MDR proteins (P-gp, TOPO-II, TS, and GST-π) in primary tumors and lymph node metastases in ESCC patients, differences in expression of those proteins have been demonstrated in a variety of primary tumors. This suggests that those four proteins could still be clinically significant. In the current study, for example, all patients expressed GST-π in both the primary and secondary cancer sites. Additional research may therefore demonstrate that chemotherapy protocols involving agents that inhibit GST-π should not be used in patients with ESCC. It is possible that additional research will show that chemotherapeutic protocols involving medications that are sensitive to the MDR effects of GST-π should not be used in patients with ESCC such as cyclophosphamide, mustard nitrogen, and clorambucils (paclitaxel, cisplatin, 5-fluoracil, and irinotecan). This hypothesis is based on the fact that of the currently available chemotherapy regimens for ESCC (that commonly include cyclophosphamide, mustard nitrogen, and chlorambucils), none can reduce the expression of GST-π. GST-π degrades drugs via catalysis, which may reduce the cytotoxicity of chemotherapeutics, resulting in resistance. Thus, it is possible that additional studies may ultimately demonstrate that drugs aiming to reduce GST-π expression may be beneficial. Further research in this field is certainly warranted to identify an "ideal" chemotherapeutic strategy for patients with ESCC.

The primary limitations of this study are that the sample size was relatively small and all included patients were recruited from one hospital. In addition, the specific locations of the lymph nodes were not analyzed with respect to different protein expression.

Conclusions

The results of this study demonstrate that there was little difference in the expression of the five analyzed MDR proteins between the primary tumors and the metastasized lymph nodes in the same patient, with the exception of LRP. Interestingly, all patients expressed GST-π in both the primary and secondary cancer sites, suggesting that the design and application of chemotherapeutic protocols capable of reducing GST-π expression may be beneficial for patients with ESCC.

Conflict of Interest The authors declare that no conflicts of interest exist.

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