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QUANTITATIVE ANALYSIS OF METASTATIC MICROENVIRONMENT OF ESOPHAGEAL CANCER

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ABSTRACT – *Objective:* To investigate the correlation between the expression of tumor-associated macrophages (TAM) and tumor neovascularization, as well as their impact on the prognosis of patients with early-stage esophageal cancer (EC).

Patients and Methods: A sample of 83 esophageal cancer (EC) patients was collected, and the expression of cancer cells, tumor-associated macrophages (TAMs), and tumor neo-vessels in the tumor tissue and its surrounding areas was quantified using a quantum dots-based immunofluorescence technique. The relationship between the expression of these components and the patients' clinical and pathological characteristics, as well as overall survival (OS) and disease-free survival (DFS), was then analyzed.

Results: Of the 83 EC tissues, 16 (19.28%) had low TAMs density and low microvessels density (MVD), 5 (6.02%) had high TAMs density and low MVD, 40 (48.19%) had low TAMs density and high MVD, and 22 (26.51%) had high TAMs density and high MVD. There was a statistically significant difference in MVD between different TAMs density subgroups ($p = 0.002$). High MVD risk with high TAMs density is significantly increased (OR = 0.421 [95% CI: 0.219-0.784], $p = 0.011$). A statistically significant difference in DFS was found between high and low TAMs density subgroups ($p < 0.05$), but no difference between high and low MVD subgroups ($p > 0.05$). Multivariate analysis by Cox regression indicated that both the TAMs density and MVD were the independent prognostic factors.

Conclusions: The TAMs density and MVD of tumor microenvironment in surgical specimens were strong prognosticators for EC patients. High TAMs and high MVD were associated with shorter DFS and shorter OS, respectively.

KEYWORDS: Esophagus cancer, Epithelial-to-mesenchymal transition, Tumor-associated macrophages, Microvessel density, Multiplexed quantum dot imaging.

INTRODUCTION

Esophagus cancer (EC) is a major global health concern, particularly in China, where it is a leading cause of cancer death[1-3]. The most common subtype of EC in China is esophageal squamous cell carcinoma, accounting for 90% of all cases[1-3]. Despite advances in diagnosis and therapy, the 5-year survival rate remains low due to its tendency for early metastasis[1-3]. To better understand the mechanisms of EC metastasis, researchers need to focus on the interplay between epithelial-to-mesenchymal transition (EMT) and the tumor microenvironment (TME)[4-6]. The TME plays a crucial role in tumor metastasis and is characterized by inflammation and angiogenesis[4-6]. Tumor-associated macrophages (TAMs) are the main immune cells in the TME and

are believed to contribute to inflammation and promote cancer cell migration and invasion. However, the significance of TAMs in EC patients remains a subject of debate[7-11]. Angiogenesis is critical to tumor metastasis as it provides the necessary oxygen and nutrients for cancer cells to spread[12]. Microvessel density (MVD) is a more accurate predictor of recurrence risk and can provide a better understanding of the heterogeneity of angiogenesis within different tumor types[12-14]. Further research is needed to understand the interactions among EC cells, TMAs, and neovessels in the TME and their prognostic value. Quantum Dots-Based Multiplexed Imaging allows for a comprehensive understanding of the intricate interactions between tumor cells and their surrounding environment, thus leading to improved insights into cancer progression and prognosis[16-18]. Meanwhile, its ability to accurately quantify and characterize the tumor microenvironment in various types of cancers[16-18]. In this study, quantum dot multicolor imaging technology was applied to esophageal cancer (EC) surgical tissue samples to investigate the spatial relationship between infiltrating tumor-associated macrophages (TAMs) and neovascularization in the tumor microenvironment (TME) of EC. It was discovered that a high density of TAMs and high microvessel density (MVD) were linked to poorer prognosis, along with shorter disease-free survival and overall survival. This research introduces new prognostic markers for EC and supports the pursuit of more personalized treatment strategies to enhance patient survival rates.

PATIENTS AND METHODS

Patients and follow-up

A total of 83 patients diagnosed with EC who underwent surgery between February 2016 and February 2022 were analyzed in the study. The tumor, node, metastasis (TNM) stage was assessed using the 8th edition of the American Joint Committee on Cancer TNM staging[15]. At the time of the last update on October 1, 2022, the median follow-up time was 46.3 months, with a range of 8.6 to 83.1 months. Out of these 83 patients, 44 (53.0%) had passed away. The overall survival (OS) was calculated as the time from the date of surgery to death. The disease-free survival (DFS) was calculated as the time from the date of surgery to the first recurrence or until the date of death or the last follow-up if recurrence was not detected. Recurrence was determined based on the results of endoscopic biopsy, ultrasonography, or CT scans with positive peritoneal cytology, chest radiography, and radiography or bone scans. If the recurrence occurred within the abdominopelvic region, it was considered local-regional recurrence, while recurrence through blood flow was considered distant metastasis, such as liver and lung metastasis. The study was approved by the Medical Ethics Committee of the Chongqing University Cancer Hospital on February 03, 2016 (Grant No. CZLS2023085-A-1) and was conducted following the Declaration of Helsinki. All patients provided written informed consent before participating in the study.

Tissue microarray and QDs-based multiplexed molecular imaging

Tissue microarray (TMA) analysis in this research referred to literature[16-18]. The primary antibodies were mouse anti-pan CK (ab7753, Abcam, London, UK, dilution 1/100), rabbit anti-CD105 (Ab231774, Abcam, London, UK, dilution 1/100), and rat anti-CD68 (ab53444, Abcam, London, UK, dilution 1/100). The QDs probes were secondary antibodies conjugated with QDs on the $F(ab')_2$ Fragments including QDs-525 probe (QDs-525 goat $F(ab')_2$ anti-mouse IgG conjugate, Q11621MP, Thermo Scientific Pierce Products, Waltham, MA, USA, dilution 1/200), QDs-585 probes (goat $F(ab')_2$ anti-rabbit IgG conjugate, Q11411MP, Thermo Scientific Pierce Products, dilution 1/200), QDs-655 probe (QDs-655 goat $F(ab')_2$ anti-rat IgG conjugate, Q11041MP, Thermo Scientific Pierce Products, dilution 1/200).

Evaluation of immunofluorescent findings

The slides were analyzed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) in conjunction with an Olympus DP72 camera and CRi Nuance multispectral imaging systems. The QDs-525, QDs-585, and QDs-655 were all excited using ultraviolet light in the range of 330-385 nm. Images were captured at a low magnification (x100) with high resolution using the DP-BSW software, ensuring identical settings were applied to each image to prevent bias. Esophageal cancer (EC) cells were labeled green, with an emission spectrum of 655 nm; neovessels were labeled orange, with an emission spectrum of 585 nm; and macrophages were labeled red, with an emission spectrum of 655 nm. The number of infiltrating macrophages

was counted in each core, and the average of two cores from the same patient was taken as the total count for further analysis. The same method was employed to count neovessels, considering only those with a diameter of less than eight erythrocytes. This criterion was based on CD105 being a specific marker for newly formed and activated small blood vessels.

Statistical analysis

Statistical analyses were conducted using SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA). Cumulative survival was computed using the Kaplan-Meier method and analyzed with the Log-rank test. A secondary analysis was undertaken to examine the relationship between immunohistochemical variables and clinicopathological characteristics. For the comparison of individual variables, Fisher's exact test, *t*-test, and Mann-Whitney test were utilized as appropriate. A two-tailed *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Major clinico-pathological features of EC patients and histological findings

In this study, of the 83 cases analyzed, 58 (69.88%) were male and 25 (30.12%) were female. Table 1 summarizes the demographic and clinicopathological characteristics, including tumor location, pathological types, TNM stage, and treatments. TAMs and tumor neovascularization predominantly reside in the extracellular matrix at the junction between the cancer and the interstitium. Notably, a significant presence of TAMs and neo-vessels is observed at the leading edge of cancer tissue invasion in both well-differentiated and poorly differentiated EC tissues. In contrast, infiltrating TAMs and neovascularization are scarcely seen in

Table 1. Patients demographics and clinico-pathological characteristics (n=83).

Parameter	Value (%)
Age	56.9±13.6
Gender	
Male	58 (69.88%)
Female	25 (30.12%)
Tumor location	
Cervical esophagus	0 (0.00%)
Upper thoracic esophagus	28 (33.73%)
Middle and lower thoracic esophagus	55 (66.26%)
Tumor size (cm^2)	
Median (range)	18 (0.35-155)
Serosa invasion	
Yes (%)	28 (33.73%)
No (%)	55 (66.26%)
Lymph node metastasis	
Yes (%)	56 (67.47%)
No (%)	27 (32.53%)
Distant metastasis	
Yes (%)	0 (0.00%)
No (%)	83 (100.00%)
TNM stages	
Early (stages I, II)	74 (89.16%)
Advanced (stages III, IV)	9 (10.84%)
Chemotherapy	
Yes (%)	38 (45.78%)
No (%)	45 (54.22%)

peritumoral normal tissues. EC cells, whether clustered or scattered, show clear nuclear division images in areas rich in TAMs and neo-vessels within the cancer nest. This indicates that the invasion of TAMs and the development of tumor blood vessels (angiogenesis) are crucial to EC invasion and dissemination. Moreover, angiogenesis within tumor tissues appears to occur in proximity to infiltrating TAMs, suggesting a potential link between TAMs density and MVD. To better understand the variation in the microenvironment of tumor and peritumoral tissues, a quantitative analysis was performed to examine the levels of TAMs and new blood vessels (neovessels) in EC tissue. The 83 tumor tissue samples were categorized into four groups based on the presence of tumor stromal features (TAMs density and MVD) (as shown in Figure 1 and Table 2).

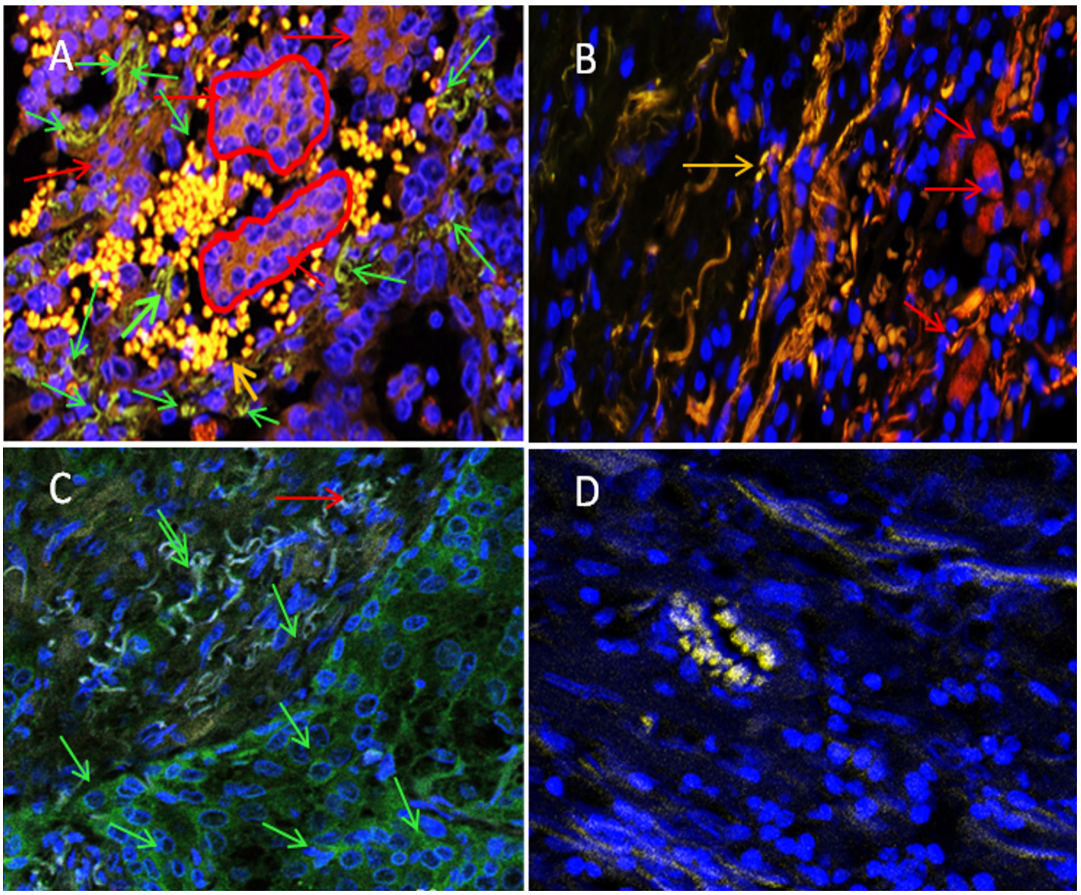

Figure 1. Infiltrating TAMs in relation to MVD. (A) High TAMs density and high MVD. (B) Low TAMs density and high MVD. (C) High TAMs density and low MVD. (D) Low TAMs density and low MVD were found in differentiated esophageal squas cell carcinomas. Red arrow: neovascularization; Green arrows: TAMs; Yellow arrow: Cancer cells.

Table 2. The TAMs infiltration and MVD distribution.

Groups	Tumoral tissue (n=83)	Peritumoral tissue (n=21)	*p*-value
TAMs density (%)			$p < 0.05$
Low (%)	56 (67.47%)	19 (90.48%)	
High (%)	27 (32.53%)	2 (9.52%)	
MVD (%)			$p < 0.001$
Low (%)	19 (22.89%)	18 (85.71%)	
High (%)	64 (77.11%)	3 (14.26%)	

Of the 83 tumor tissues, 27 (32.53%) showed high TAMs density, and 64 (77.11%) had high MVD. In contrast, among the 21 distant peritumoral normal tissues, only 2 (9.52%) had high TAMs density and 3 (14.26%) had high MVD. The results showed that both TAMs density and MVD were higher in the tumor tissues compared to the peritumoral tissues ($p < 0.05$ and $p < 0.001$, respectively). After exploring the role of TAMs in angiogenesis and neovessels maturity, the impact of these stromal features on EC progression was evaluated. Table 3 shows that several traditional clinicopathological characteristics, including tumor size, T stage, LN metastasis, serosa invasion, distant metastasis, TNM stage, recurrent status, and the positive rate of LN resection, were associated with overall survival (OS) in a univariate analysis. However, neither macrophage density nor MVD was found to be related to sex or lymph node metastasis.

Table 3. Relationship between TAMs density/MVD and clinico-pathological features.

Variable	TAMs		*p*-value	MVD		*p*-value
	Low (n=56)	High (n=27)		Low (n=19)	High (n=64)	
Age (year)	59.12±7.25	56.2±10.05	NS	55.3±11.57	61.2±7.32	NS
Gender						
Male	40 (71.4%)	18 (66.67%)	NS	16 (84.21%)	42 (65.62%)	0.032*
Female	16 (28.6%)	9 (33.33%)		3 (15.79%)	22 (34.38%)	NS
Tumor location						
Cervical esophagus	0	0	NS	0	0	NS
Upper thoracic esophagus	19 (33.93%)	9 (33.33%)		6 (31.58%)	22 (34.38%)	
Middle and lower thoracic esophagus	37 (66.07%)	18 (66.67%)		13 (68.42%)	42 (65.62%)	
Tumor size (cm^2)	17.3 (0.35-128)	19.7 (1.05-132)	NS	12.3 (0.75-109)	20.9 (2.15-155)	0.026*
Serosa invasion			NS			0.038*
Yes (%)	17 (30.36%)	11 (40.74%)		13 (68.42%)	15 (23.44%)	
No (%)	39 (69.64%)	16 (59.26%)		6 (31.58%)	49 (76.56%)	
Lymph node metastasis			NS			0.045*
Yes (%)	38 (67.85%)	18 (66.67%)		10 (52.63%)	46 (71.88%)	
No (%)	18 (32.14%)	9 (33.33%)		9 (47.37%)	18 (28.12%)	
TNM stages			NS			
Early (stages I, II) (%)	50 (89.29%)	24 (88.89%)		14 (73.68%)	60 (93.75%)	0.040*
Advanced (stages III, IV) (%)	6 (10.71%)	3 (11.11%)		5 (26.32%)	4 (6.25%)	
Recurrence			0.007*			NS
Yes (%)	8 (14.29%)	8 (29.63%)		3 (15.79%)	13 (20.31%)	
No (%)	48 (85.71%)	19 (70.37%)		16 (84.21%)	51 (79.69%)	
Recurrence site			NS			NS
Loco-regional	8 (14.29%)	5 (18.51%)		4 (21.06%)	9 (14.06%)	
Distant	2 (3.57%)	1 (3.70%)		1 (5.26%)	2 (3.12%)	

*$p < 0.05$ was considered as statistically significant; Abbreviation - NS: Not significant.

The results indicated that patients with high TAMs density appeared to have a higher rate of recurrence ($p = 0.007$), and those with high MVD had larger tumor sizes ($p = 0.026$). However, there was no statistically significant difference in the recurrence rate between the high and low MVD subgroups ($p = 0.224$). A linear correlation analysis further revealed that the risk of high MVD was significantly increased in patients with high TAMs density (odds ratio [OR] = 4.861 [95% CI: 1.652–11.983], $p = 0.002$; Logistic Regression) (Table 4). As stromal features of tumor envirommment, TAMs density and MVD were strong prognosticator for EC. To examine the prognostic significance of tumor-associated macrophages (TAMs) density and microvessel density (MVD) in esophageal cancer (EC) patients, this study conducted a prognostic analysis to explore the relationships between TAMs density and MVD with overall survival (OS) and disease-free survival (DFS). As presented in Figure 2 and Table 5, the median OS for the 83 patients was 39.0 months (range 0.9 to 83.1), with 1-year, 3-year, and 5-year survival rates of 83%, 59%, and 42%, respectively. The median OS was 39.2 months (range 0.9-80.1) in the low TAMs density group and 21.4 months (range 5.5-83.1) in the high TAMs density group. For the MVD, the median OS was 40.3 months (range 0.9-80.1) in the low MVD group and significantly higher in the high MVD

Table 4. The relationship among the TAMs density and MVD.

Variable		MVD		*p*-value
		low	high	
TAMs	Low	16 (19.28%)	40 (48.19%)	0.002*
	High	5 (6.02%)	22 (26.51%)	

*$p < 0.05$ was considered as statistically significant.

group at 54 months, with 77.1% surviving. A statistically significant difference in OS was observed between the MVD subgroups ($p = 0.0214$), but no significant difference was found between the TAMs density subgroups ($p > 0.05$). The median DFS of 83 patients undergoing radical resection was 36.2 (range 0.9 to 83.1) months, with DFS rates of 84%, 71%, and 60% at 1, 3, and 5 years, respectively. The median DFS was 36.5 (0.9-80.1) in low TAMs density group, 14.5 (5.5-83.1) in high TAMs density group, 40.3 (5.5-83.1) in low MVD group and 42.2 (0.9-80.1) in high MVD group, respectively. A statistically significant difference in DFS was found between TAMs density subgroups ($p = 0.001$), but no difference between MVD subgroups ($p = 0.0038$) (Figure 2, group B; Table 5).

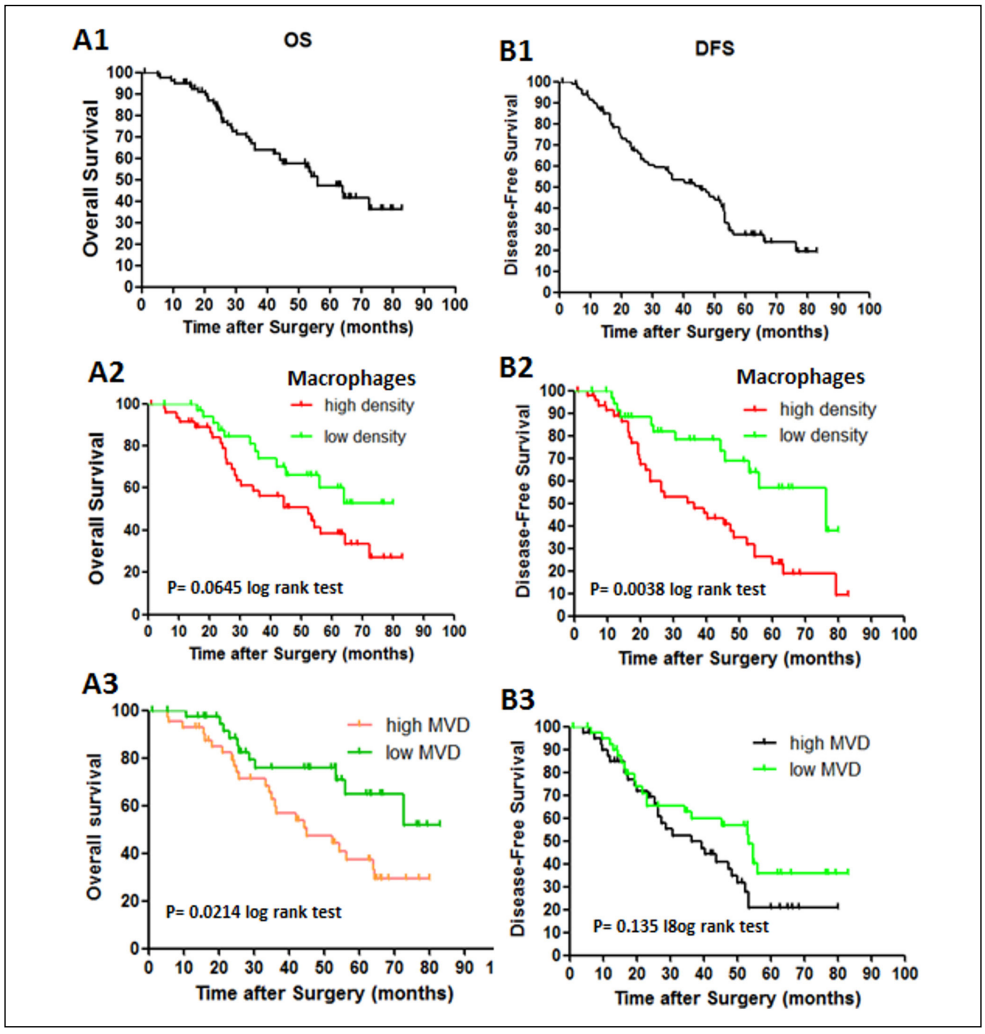

Figure 2. Cumulative OS and DFS curves of esophageal cancer patients. (A1, B1) OS and DFS in patients with esophageal cancer. (A2, B2) High macrophage density is not associated with an increased risk of death in patients with esophageal cancer, but is associated with an increased risk of recurrence. (A3, B3) High MVD linked to poor overall survival, but not to relapse risk.

Table 5. Relationship between TAMs density and MVD.

Variable		OS months (median range)	*p*-value	DFS months (median range)	*p*-value
TAMs density	Low (n=56)	39.2 (0.9-80.1)	*P* = 0.0645	36.5 (0.9-80.1)	*P* = 0.0038*
	High (n=27)	20.4 (5.5-83.1)		14.5 (5.5-83.1)	
MVD	Low (n=29)	40.3 (0.9-80.1)	*P* = 0.0214*	40.3 (5.5-83.1)	*P* = 0.1352
	High (n=54)	21.4 (5.5-83.1)		42.2 (0.9-80.1)	

*$p < 0.05$ was considered as statistically significant.

Both univariate and multivariate analyses were carried out. Results from univariate analysis revealed a correlation between MVD and OS. It was discovered that individuals with high MVD had a higher chance of death as compared to those with low MVD (HR = 1.788 [95% CI: 1.331-3.184], $p = 0.026$). On the other hand, DFS analysis showed that tumor size, positive rate of resected LN, macrophage density, and neovessels maturity had an impact on DFS. Those with a high density of TAMs were found to have a 110% higher risk of relapse (HR = 2.212 [95% CI: 1.443 to 3.435], $p < 0.001$). To identify the most influential factors, the results from univariate analysis were incorporated into the multivariate Cox proportional risk analysis. The results indicated that MVD (HR = 2.343 [95% CI: 1.421-4.343], $p = 0.0095$) was an independent predictor of OS after controlling for other variables. Additionally, the density of infiltrating TAMs (HR = 2.321 [95% CI: 1.231-4.532], $p < 0.001$) was found to be an independent predictor of DFS in multivariate analysis.

DISCUSSION

EC invasion and metastasis remain the root cause of mortality[1-3]. The change in tumor microenvironment is the main factor of EC cell invasion and metastasis, especially the dynamic interaction between cancerous lesions and their surrounding matrix[19-21]. Despite its significance, a thorough understanding of these interactions has been elusive due to the paucity of technological platforms capable of concurrently examining these complex phenomena[22-24]. Currently, multiplexed quantum dot imaging can directly visualize the interaction of malignant cells and their microenvironment in human carcinomas, thereby providing a more complete picture of the intricate and dynamic biological events associated with cancer invasion[22-24]. In this study, multiplexed quantum dot imaging was utilized to reveal the principal elements involved in the critical steps of EC invasion simultaneously, yielding novel spatiotemporal information regarding the interactions between the EC and its microenvironment. The study investigated the significance of tumor microenvironment in the prognosis of EC patients. A total of 83 EC patients were analyzed to evaluate the role of invasive macrophages (TAMs) density and MVD in their prognosis. The primary clinical parameters used to assess the value were overall survival and disease-free survival. The findings were as follows: first, this study used a new imaging technique to examine the interactions between cancer cells and the microenvironment in human carcinomas. The results showed that the presence of TAMs and tumor neovascularization played a role in the cancer invasion and metastasis process[4-6]. Therefore, we speculate that macrophages and neovascularization are jointly involved in the process of local invasion of cancer cells, that is, the invasion of cancer cells is not the behavior of single individual cells, but the process of local invasion and metastasis is completed with the help of heavy pressure components of the tumor microenvironment[25,26]. In short, tumor cells, macrophages, and new blood vessels work together to create a well-planned environment that favors cancer metastasis and recurrence[4-6,25,26].

Secondly, our study found a correlation between the density of TAMs and MVD in tumors. The density of TAMs and MVD played a role in EC progression and prognosis, with TAM density being linked to disease-free survival but not overall survival. At the same time, we found that high TAM density increased the risk of relapse. The findings align with previous studies that have shown TAMs are present at various stages of tumor development and play a role in tumor cell invasion and survival[27-29]. TAMs play a role in promoting tumor progression by infiltrating the tumor, promoting angiogenesis, and normalizing/abnormalizing tumor blood vessels. This creates a vicious cycle between TAMs, neovascularization, and cancer cell progression[27-29].

The prediction of EC prognosis has been a highly researched topic for a long time. Historically, the focus has been on conventional clinicopathological features such as tumor size, lymph node involvement, TNM stage, and treatment approach, as well as the influence of molecular features of the cancer cells, such as EGFR and Ki-67,

on prognosis[30-32]. This study supports the significance of these traditional prognostic indicators. However, since EC is perceived as a dynamic disease, the behavior of EC cells is not solely determined by them but is greatly impacted by the tumor microenvironment[33]. Thus, it is crucial and appropriate to evaluate the prognostic value of the tumor microenvironment in EC. This study also found that while MVD is not related to DFS, it is related to the OS. Kaplan-Meier and Cox regression analyses showed that TAMs density was not an independent prognostic factor for OS, but MVD was a strong one for OS. This may suggest that tumor-infiltrating macrophages have a synergistic effect with tumor angiogenesis to promote cancer progression and that the mechanism is to promote the spread of cancer cells through immature new blood vessels and increase TAMs infiltration, even in the early stages of cancer development[25-33]. In short, tumor microenvironment, including the presence of invasive macrophages (TAMs) and MVD, plays a crucial role in the progression and prognosis of esophageal cancer (EC) patients[25-34]. The results showed that patients with high TAM density and high MVD had the worst prognosis, while those with low levels of both had the best prognosis. The composite stromal characteristics of the tumor microenvironment had more significant prognostic value than traditional clinicopathological features. The study used a new method called multiplexed quantum dot imaging to visualize the interaction between cancer cells and their microenvironment in human carcinomas, providing a more complete understanding of the biological events associated with cancer invasion.

CONCLUSIONS

The density of TAMs and MVD in the tumor microenvironment of surgical specimens were identified as strong prognosticators for patients with EC. High levels of TAMs and MVD were each associated with shorter disease-free survival (DFS) and overall survival (OS), respectively. These findings highlight a significant correlation between the characteristics of the tumor stroma, whether positive or negative, and the clinical outcomes for patients with epithelial carcinoma. The results underscore the critical importance of the tumor microenvironment in cancer progression and emphasize the need for further comprehensive research in this field.

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Ethics approval and consent to participate:
The study was approved by the Medical Ethics Committee of the Chongqing University Cancer Hospital on February 03, 2016 (Grant No. CZLS2023085-A-1) and was conducted following the Declaration of Helsinki.

Informed consent:
All patients provided written informed consent before participating in the study.

Authorship contribution statement:
Li Tang, Li Yin and Guo Qishuai: Conceptualization, Methodology, Investigation, Writing - original draft. Yanfei Mu, Chunbo Fan, Qian Luo: Investigation, Software. Li Tang, Li Yin, Qingming Jiang: Investigation, Validation, Resources. Li Tang, Li Yin and Guo Qishuai: Formal analysis, Data curation, Visualization, Writing - review & editing, Funding acquisition, Supervision, Funding acquisition.

Conflict of interest:
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Sharing Statement:
All relevant data are available upon request from the corresponding author.

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