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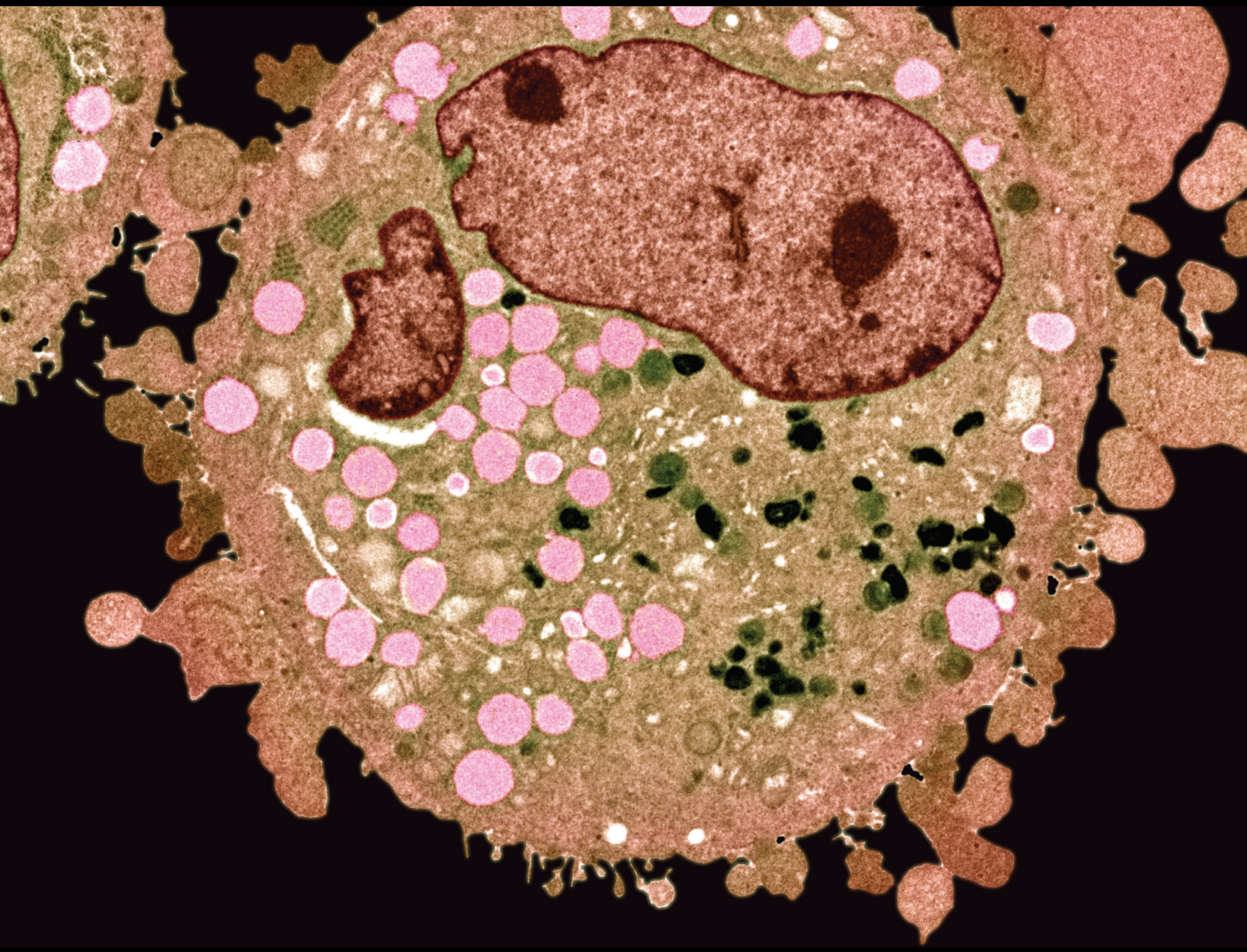

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Research Article

The Expression and Prognostic Value of FGF2, FGFR3, and FGFBP1 in Esophageal Squamous Cell Carcinoma

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Background. Esophageal squamous cell carcinoma was treated by operation and chemoradiotherapy. However, the prognosis of most patients is poor after treatment, and most studies have shown that FGF2 and its receptor (FGFR) are involved in the development of various malignant tumors. FGF2 plays an important role in tumor progression and malignancy. In this study, the immunohistochemistry of FGF2, FGFR3, and FGFBP1 was used to further verify the expression of the three proteins in 172 patients with esophageal squamous cell carcinoma (ESCC) who had not received preoperative chemoradiotherapy and its effect on the prognosis of ESCC. *Methods*. (1) χ^2 test was used to analyze the relationship between proteins and clinicopathological parameters. Survival analysis was used to investigate the effect of three proteins on prognosis. (2) Paired sample t-test was used to analyze the mRNA expression of the three proteins in fresh ESCC tissues and adjacent normal tissues. *Results*. FGF2 was correlated with tumor size ($p = 0.026$), gender ($p = 0.047$), and lymph metastasis ($p = 0.007$) in ESCC tissues. The high expression of FGFR3 was associated with tumor differentiation ($p = 0.043$ and $p < 0.05$), lymph node metastasis ($p = 0.078$ and $p < 0.1$), and race ($p = 0.033$ and $p < 0.05$). The high expression of FGFBP1 was significantly associated with the degree of tumor differentiation ($p = 0.012$), age ($p = 0.045$), and lymph node metastasis ($p = 0.032$) of ESCC patients. The expression of FGF2, FGFR3, and FGFBP1-mRNA in ESCC tissues was significantly higher than that in adjacent tissues ($p < 0.001$, $p < 0.001$, and $p = 0.001$). Patients with high expression of FGF2, FGFBP1, and FGFR3 had poor prognosis. There was a weak positive correlation between FGF2 and FGFBP1, as well as FGFR. *Conclusion*. The FGF2-FGFR3 axis may promote the progression of esophageal squamous cell carcinoma. The FGF2-FGFR3 axis may be a new direction of targeted therapy for esophageal squamous cell carcinoma. FGF2 and FGFR3 may be used as prognostic markers of esophageal squamous cell carcinoma.

1. Introduction

Esophageal cancer is a common digestive tract malignant tumor, which in sorted by histological type includes esophageal squamous cell carcinoma and esophageal adenocarcinoma. In European and American countries, the pattern is approximately 70% Barrett esophageal adenocarcinomas; while in China, the pattern is 95% esophageal squamous cell carcinoma [1], of which the 5-year survival rate is only 5%–13% [2]. There are obvious regional and national differences in the distribution of esophageal cancer in China. The high-incidence area of esophageal cancer is mainly distributed in the north China area, Dabie Mountain area, and between Fujian and Guangdong coastal area, and Xinjiang is also one of the high-risk areas [3]. The mechanism that occurs in the development of esophageal cancer is a complex combination with interactions at multistage, of multiple factors, and between multiple genes [4, 5]. Treatment of esophageal cancer should be the use of surgery and chemoradiotherapy; however, due to the late detection of most patients, the prognosis is poor after treatment.

Growth factor fibroblast growth factor-2 (FGF2), also known as basic FGF [6], has been shown to exist in low molecular and high molecular weight isomers that are

translated by a single common mRNA through another translation initiation codon [7]. Low molecular weight FGF2 is an 18 kDa protein translated from the traditional AUG initiation codon. LMW FGF2, which exists in cytoplasm and nucleus, can also be secreted by the target cells. In order to start signal, the compound of LMW, FGF2, cell surface heparin sulfate proteoglycans (HSPGs), and fibroblast growth factor receptor (FGFR) activates downstream signaling pathways, including Ras, Raf, MAPK, and ERK [8]. The high molecular weight (HMW) FGF2 subtype is initiated by the translation of the upstream CUG locus and AUG codon frame. HMW FGF2 was located in the nucleus, and the signal was independent of FGFR [9]. The downstream signaling pathways are mediated by Ras/ERK and phosphoribosyl kinase (PI3K)/AKT signaling pathways in order to promote cell mitosis and regulate cell proliferation, differentiation, and migration [10]. FGF2 also plays an important role in tumor progression and malignancy, such as breast cancer and oral squamous cell carcinoma. FGF2, regulating CSCs through Mek/Erk signaling, is an important factor in esophageal squamous cell carcinoma [11].

FGFR, FGF2 receptors, is made of three Ig-like domains in the extracellular region, a single spanning transmembrane domain, and a split tyrosine kinase domain in the cytoplasmic region [12]. As FGF binds to FGFRs, the tyrosine kinase domain in the cytoplasmic region of the receptors is activated and generates signal paths, such as the Ras-MAPK, PI3K-AKT, and PLC-γ-PKC pathways to induce cell proliferation, differentiation, migration, and tumor formation [13]. FGFR3 has carcinogenic activity in several cancers. The increased or mutated expression in FGFR3 leads to malignant progression in bladder cancer, colon cancer, and multiple myeloma [14–17].

FGFBP1 can bind to fibroblast growth factors such as FGF2, protect FGF2 from degradation, and present it to its high-affinity cell surface receptor, thus promoting the biological function of FGFs. FGFBP1 was reversibly combined with the acidic and basic fibroblast growth factor. FGF2 can closely bind HSPG in ECM and is only released through the action of FGFBP1 [18]. FGF binding protein is the key to FGF bioavailability regulatory factors [19]. Increasingly study has shown that FGFBP1 is highly expressed in skin cancer [20, 21], but not clear in the mechanism of esophageal cancer.

The FGF/FGFR tyrosine kinase signal pathway regulates multiple biological events during embryogenesis and functions in the maintenance and repair of adult tissues [22]. This pathway is also implicated in both tumorigenesis and the development of chemoresistance in various types of cancers [23].

This study is designed to preliminarily analyze the expression of FGF2, FGFR3, FGFBP1, and their relationships with clinicopathological parameters in ESCC. The correlation among these proteins was analyzed through Spearman correlation analysis. Furthermore, we investigated to evaluate the effect of FGF2, FGFR3, and FGFBP1 on the prognosis of ESCC through Kaplan–Meier analysis. Our findings suggested that the FGF2-FGFR3 axis may be a new direction of targeted therapy for ESCC.

2. Materials and Methods

From January 2014 to June 2018, 172 cases of esophageal squamous cell carcinoma (including 94 cases of Han nationality and 78 cases of Hazak nationality) were collected from the First Affiliated Hospital of Xinjiang Medical University, including their clinicopathological data: age (<65 and ≥65), gender (male and female), nationality, location (upper, middle, and lower), tumor size (<3 cm and ≥3 cm), differentiation degree (high differentiation, middle differentiation, and low differentiation), lymph node metastasis (yes and no), vascular infiltration (yes and no), nerve infiltration (yes and no); distant metastasis (yes and no), pTNM stage (the eighth edition) (IB, IIA+B, and IIIA+B+C), and treatment (surgery and postoperative chemoradiotherapy) (Table 1). None of the selected patients completed neoadjuvant therapy preoperatively. All the selected patients were patients with advanced esophageal squamous cell carcinoma without early cancer (T1N0M0). All the selected patients underwent radical resection of esophageal carcinoma and lymph node dissection. According to 2020 CSCO esophagus cancer diagnosis and treatment guidelines, the patients of cT1b cT2 N+ or cT3-cT4a, any N needs to be radical surgery and at the same time chemoradiotherapy. All the selected patients in this study required postoperative chemoradiotherapy. And the study was approved by Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University. Our follow-up time ended in July 2019 through inquiring the medical records and telephone calls.

2.1. Immunohistochemistry. (1) Anti-FGFR3 (product no. BM5016), anti-FGF2 antibody (EP1735) (ab92337), and anti-FGFBP1 antibody (ab238155) were used as the reagents

(2) *Methods*: with SP method, 172 cases of embedded esophageal cancer, paraffin tissue, and normal mucosal tissue were made into tissue chips, which were made into 4 mic continuous sections. The tissues were surgical samples. After dewaxing and dehydration, the tissue chips were put into a boiling repair solution, citric acid (PH6.0), heated to 95°C, and kept them in the acid for 20 minutes. After 30-minute cooling at room temperature, these chips were added into endogenous peroxidase and incubated for 20 minutes in the room temperature. The tissue chips were washed in phosphate-buffered saline for three-times (3 min/time). After that, anti-FGFR3 (BM5016) (1 : 50, 4°C overnight), anti-FGFBP1 antibody (ab238155) (1 : 800, 4°C overnight), and anti-FGF2 antibody (EP1735) (ab92337) (1 : 800, 4°C overnight) were dipped onto these chips separately. When the time is up, the chips were washed in phosphate buffer saline (PBS) for three times, were dropped with goat anti-mouse secondary antibody (PV-6002, Zsbio, Beijing, China), and were placed in an oven at 37°C for 40 minutes. Finally, the slides were dyed in prepared DAB solution, redyed with hematoxylin, dehydrated with graded alcohol, covered with slide, and installed for review.

(3) The staining intensity score of FGFBP1 was 0 (negative), 1 (weak), and 2 (strong). The dyeing range is based on the percentage of positive tumor cell score of 0 (negative), 1 (1% and 25%), 2 (26% and 50%), 3 (51% and 75%), or 4

Table 1: Clinicopathological characteristics of esophageal squamous cell carcinoma patients (n (%)).

Characteristics and finding	$n = 172$
Age (years old)	63.53 (38-83)
Tumor size (cm)	3.81 (1-8.5)
Gender	
Male	129 (75)
Female	43 (25)
Race	
Han	94 (54.7)
Kazak	78 (45.3)
Tumor site	
Upper	8 (4.7)
Middle	95 (55.2)
Lower	69 (40.1)
Differentiation	
Well	33 (19.2)
Moderate	97 (56.4)
Poor	42 (24.4)
pTNM	
IB	12 (7)
IIA,B	86 (50)
IIIA,B,C	74 (43)
Lymph metastasis	
Negative	113 (65.7)
Positive	59 (34.3)
Vessel invasion	
Negative	141 (82)
Positive	31 (18)
Nerve invasion	
Negative	136 (79.1)
Positive	36 (20.9)
Distant metastases	
Negative	132 (76.7)
Positive	40 (23.3)
Treatment	
Surgery	109 (63.4)
Postoperative chemoradiotherapy	63 (36.6)
FGF2	
Low expression	55 (32)
High expression	117 (68)
FGFR3	
Low expression	61 (35.5)
High expression	111 (64.5)
FGFBP1	
Low expression	60 (34.9)
High expression	112 (65.1)

(76% and 100%). The final score is the product of the staining intensity score and the staining range score. If the final score is 0 to 4(±), the case is ultimately considered negative. If the final score is 5(+) to 8(+++), the final score is positive. Expression levels of FGF2 and FGFR3 were assessed by semiquantitative scoring, including percentage of total lesion area (0-100%) and staining intensity (0-3). The expression of epithelium, endothelial cells, and stroma was analyzed in all cases. The classification of area positive rate is as follows: <10% = 0, 10-25% = 1, 25-50% = 2, 50-75% = 3, and >75% = 4. To assess intensity, the grades were as follows: 0: none; 1: mild; 2: moderate; and 3: strong staining. The percentage score (0–4) was multiplied by the intensity score (0–3), and the final score was assigned with 0–4 for negative staining and 5–12 for positive staining [24]

Table 2: PCR primer sequences.

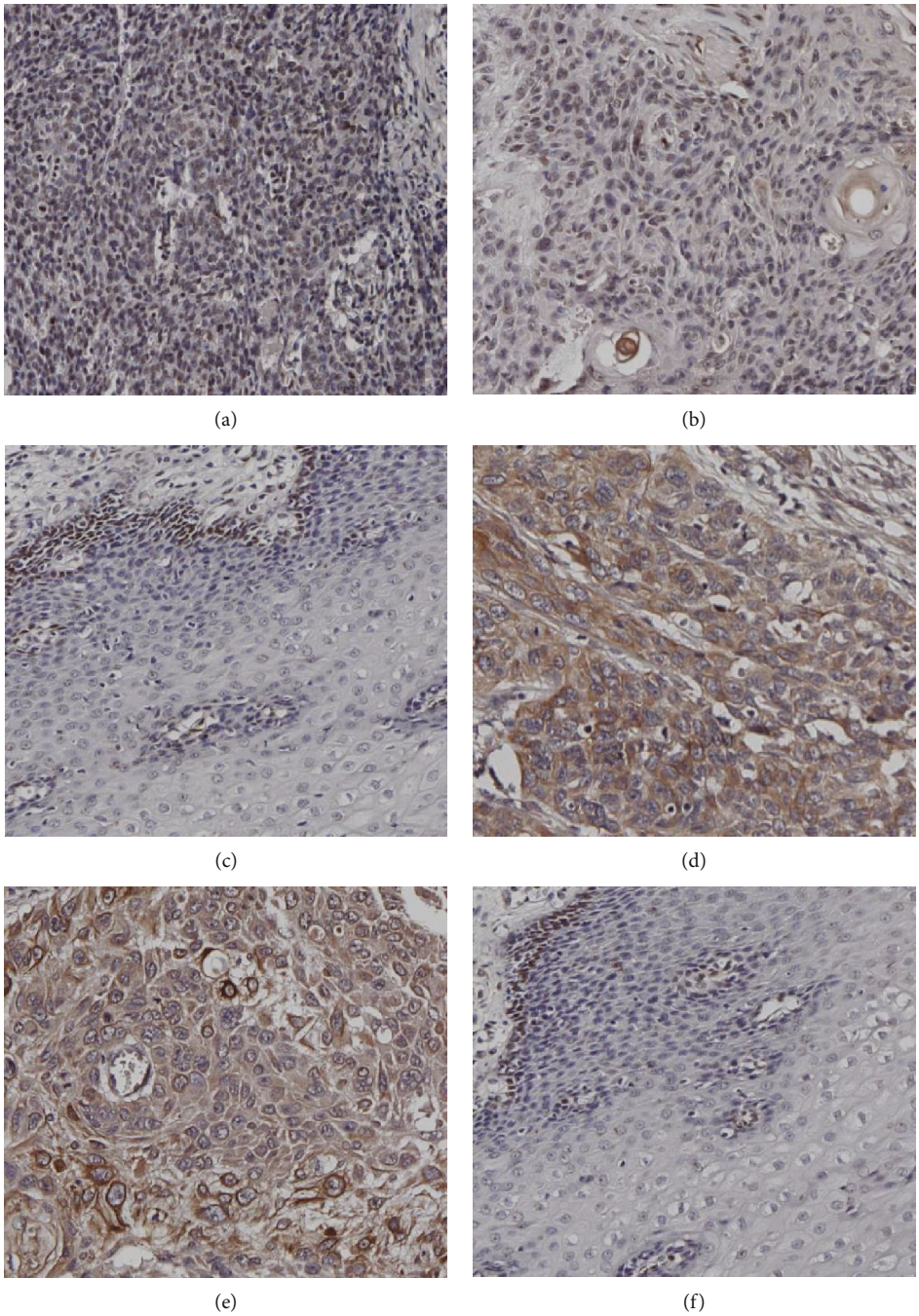
Gene name		Sequence
FGF2	Forward	TTCAAGCAGAAGAGAGAGGAG
	Reverse	TCCGTAACACATTTAGAAGCC
FGFR3	Forward	ACCAAGCCTGTCACCGTAG
	Reverse	CAGAAACTCCCGCAGGTTACC
FGFBP1	Forward	GGGAGGAGCTGTGAGTAACG
	Reverse	CAGGCAGTGCGAGTGAATTG

2.2. qRT-PCR. (1) *Extraction of total RNA*: firstly, 29 cases of esophageal cancer and their paired adjacent normal tissues were taken out from the refrigerator at -80°C. Secondly, the liquid nitrogen was added to them for milling, and Trizol reagent was also added to extract the total RNA in the tissues after grinding according to the instructions. Thirdly, the concentration of total RNA in the extracted tissues was measured by NanoDrop 2000c uv spectrophotometer. After the electrophoresis test, cDNA was transcribed. According to the instructions of the reverse transcription kit, 2 ng taken from the total RNA extracted was added into the reverse transcription reaction system under the following conditions: 25°C 5 min, 42°C 60 min, 70°C 5 min, and 4°C forever. The synthesized cDNA was stored in a refrigerator at -80°C for later use. The primer sequences are listed in Table 2.

(2) qRT-PCR use a two-step method with SYBR Green (Applied Biosystems 7500, Thermo Fisher Scientific)

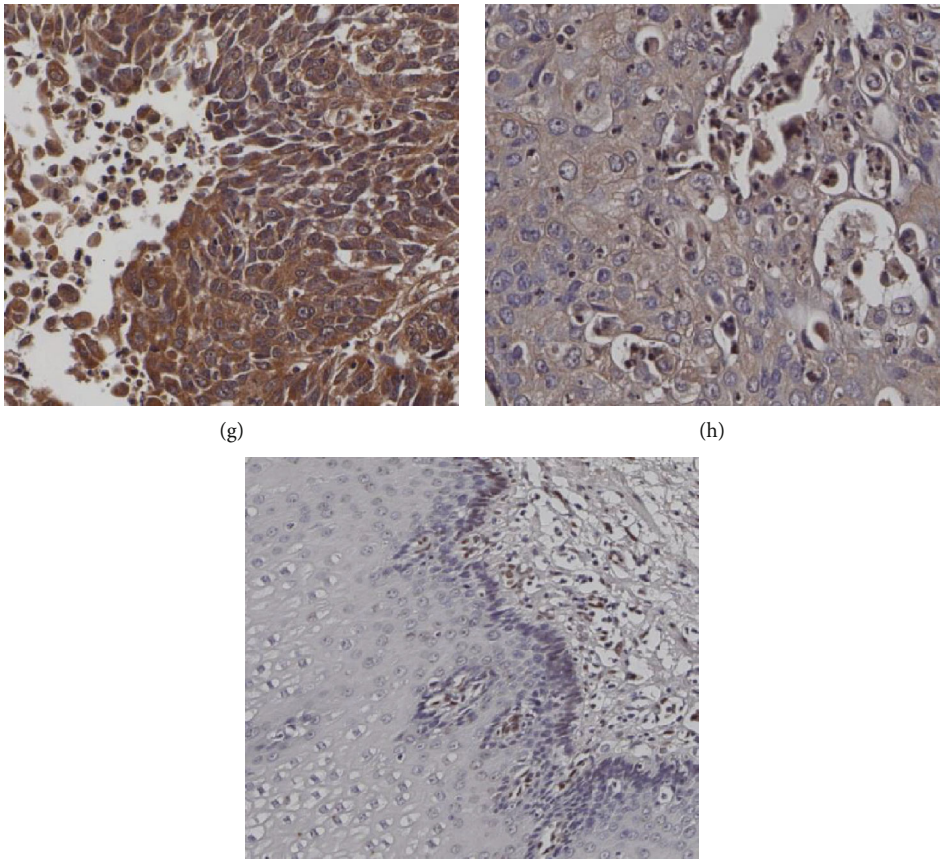
(3) The above reaction elements were added into the reaction system, and the reaction conditions were as follows: predenaturation at 95°C for 2 min, denaturation at 95°C for 10 s, annealing at 60°C for 10 s, extension at 72°C for 5 min, and 40 cycles in total. Each sample was repeated at least 3 times, with 3 multiple holes set for each time (Table 2)

2.3. Statistical Analysis. SPSS 25.0 statistical software was used. χ^2 test and Fisher exact test were used to analyze the relationship between clinicopathological characteristics and the expression of FGF2, FGFR3, and FGFBP1 in esophageal squamous cell carcinoma. The correlation between FGF2, FGFR3, and FGFBP1 was also analyzed by Spearman grade correlation. Use overall survival and progression-free survival to estimate survival time. Progression-free survival is



(a) (b) (c) (d) (e) (f)

Figure 1: Continued.



(g) (h) (i)

Figure 1: Immunohistochemical staining of FGF2, FGFR3, FGFBP1 expression in ESCC and normal esophagus mucosa. (a, b) Positive expression of FGF2 in ESCC tumor tissue; (c) positive expression of FGF2 in the basal layer of normal esophageal mucosa; (d, e) positive expression of FGFR3 in ESCC tumor tissue; (f) positive expression of FGFR3 in the basal layer of normal esophageal mucosa; (g, h) positive expression of FGFBP1 in ESCC tumor tissue; (i) negative expression of FGFBP1 in normal esophageal mucosa.

defined as the diagnosis of esophageal squamous cell carcinoma of the time to tumor progression or death. Overall survival is defined as the diagnosis of esophageal cancer patients with time to death or final follow-up time (2019-07-01). The effects of FGF2, FGFR3, FGFBP1 protein, and clinicopathological parameters on the prognosis of ESCC were analyzed by Kaplan–Meier method. Based on Kaplan–Meier analysis results, independent factors related to the prognosis of esophageal squamous cell carcinoma were further analyzed by Cox proportional risk model. The method was forward LR, and $p < 0.05$ was considered significant. For qRT-PCR results, the ct value of the cancerous tissue and the ct value of the adjacent tissues were used to calculate the $2\text{-}\Delta\Delta$ct value. If the two groups of values accord with normal distribution and homogeneity of variance, the paired sample t-test is adopted. If the measured data are nonnormal and homogeneity of variance, the nonparametric Wilcoxon rank sum test is adopted.

3. Results and Analysis

3.1. The Expression of FGF2, FGFR3, and FGFBP1 in ESCC and Their Relationship with Clinicopathological Parameters. The expression of FGF2 in ESCC is shown in the figure (Figures 1(a)–1(c)). FGF2 was positive in the nucleus and cytoplasm of ESCC, negative in normal esophageal mucosa, or positive only in basal cells. In this study, there were 172 patients with esophageal squamous cell carcinoma, of which 55 (32%) were FGF2 negative and 117 (68%) were FGF2 positive. Statistical analysis showed that high expression of FGF2 was correlated with tumor size ($p = 0.026$), gender ($p = 0.047$), and lymph metastasis ($p = 0.007$). The expression of FGF2 was not correlated with race ($p = 0.794$), age ($p = 0.053$), tumor site ($p = 0.902$), differentiation ($p = 0.231$), and pathological stage of ESCC cases ($p = 0.325$) ($p > 0.05$) and so on (Table 3).

The expression of FGFR3 in ESCC and its relationship with clinicopathological parameters was investigated. As is shown in Figures 1(d)–1(f), positive FGFR3 staining signals were brown and yellow, located in the cytoplasm and membrane of the esophageal cancer cells. In normal tissues adjacent to cancer, FGFR3 positive signals were found in the basal layer of esophageal mucosa. All tumor specimens were divided into the FGFR3-low expression group (61 cases, 35.5%) and the FGFR3-high expression group (111 cases, 64.5%). As is shown in Table 3 summary, the correlation is

Table 3: Relationship between expression of FGF2, FGFR3, and FGFBP1 in ESCC and clinicopathological parameters (n).

Characteristic	Total	FGF2 expression n		p	FGFR3 expression n		p	FGFBP1 expression n		p
	$n = 172$	Negative	Positive		Negative	Positive		Negative	Positive	
Gender				0.047			0.519			0.712
Male	129	36	93		44	85		46	83	
Female	43	19	24		17	26		14	29	
Race				0.794			0.033			0.302
Han	94	31	63		40	54		24	54	
Hazak	78	24	54		21	57		36	58	
Age (years)				0.053			0.384			0.045
<65	91	35	56		35	56		38	53	
≥65	81	20	61		26	55		22	59	
Tumor size				0.026			0.885			0.811
<3 cm	44	20	24		16	28		16	28	
≥ 3 cm	128	35	93		45	83		44	84	
Tumor site				0.902			0.548			0.457
Upper	8	3	5		4	4		4	4	
Middle	95	31	64		35	60		35	60	
Lower	69	21	48		22	47		21	48	
Differentiation				0.231			0.043			0.012
Well	33	11	22		12	21		15	18	
Moderate	97	35	62		43	54		41	56	
Poor	42	9	33		6	36		4	38	
Pathological stage				0.325			0.897			0.320
IB	12	6	6		5	7		6	6	
IIA,B	86	28	58		30	56		32	54	
IIIA,B,C	74	21	53		26	48		22	52	
Lymph metastasis				0.007			0.078			0.032
Negative	113	44	69		45	68		45	68	
Positive	59	11	48		16	43		15	48	
Vascular invasion				0.644			0.68			0.735
Negative	141	44	97		51	90		50	91	
Positive	31	11	20		10	21		10	21	
Nerve invasion				0.313			0.764			0.826
Negative	136	46	90		49	87		48	88	
Positive	36	9	27		12	24		12	24	
Distant metastases				0.142			0.494			0.718
Negative	132	46	86		45	87		47	85	
Positive	40	9	31		16	24		13	27	

between FGFR3 expression and clinicopathological features. Statistical analysis showed that overexpression of FGFR3 expression was correlated with tumor differentiation ($p = 0.043$ and $p < 0.05$), lymph node metastasis ($p = 0.078$ and $p < 0.1$), and race ($p = 0.033$ and $p < 0.05$).

The expression of FGFBP1 in ESCC is shown in Figures 1(g)–1(i). The positive signal of FGFBP1 is mainly located in the cytoplasm and membrane of the esophageal cancer cells and is positively expressed in the normal esophageal mucosal epithelium. Statistical analysis showed that the high expression of FGFBP1 was significantly correlated with the degree of tumor differentiation ($p = 0.012$), age ($p = 0.045$), and lymph node metastasis ($p = 0.032$) of ESCC patients, while no association was significantly correlated for high expression of FGFBP1 with gender ($p = 0.559$), race ($p = 0.302$), tumor size ($p = 0.267$), tumor site ($p = 0.457$), pathological stage ($p = 0.320$), vascular invasion ($p = 0.735$), and so on ($p > 0.05$) (Table 3).

3.2. Correlation of FGF2, FGFR3, and FGFBP1 Protein Expression. Spearman level correlation analysis was performed in 172 cases of esophageal squamous cell carcinoma,

and the protein expression of FGF2 was significantly correlated with FGFR3 and FGFBP1 ($p < 0.001$, rs = 0.612; $p < 0.001$, rs = 0.649). FGFR3 and FGFBP1 were further analyzed and found that there is a positive correlation ($p < 0.001$, rs = 0.656), as is shown in Tables 4 and 5. These three proteins are highly expressed in esophageal squamous cell carcinoma. According to their correlation, it was speculated that the FGF2-FGFR3 axis formed by FGF2, FGFR3, and FGFBP1 may promote ESCC progression.

3.3. qRT-PCR. The results showed that mRNA expressions of FGF2 ($p < 0.001$), FGFR3 ($p < 0.001$), and FGFBP1 ($p = 0.001$) were higher in cancer tissues than in the adjacent tissues ($p < 0.05$) (Figure 2). The difference was statistically significant. The results were consistent with the immunohistochemical results.

3.4. Prognostic Factors for OS and PFS. Kaplan–Meier method was used to investigate the relationship between protein expression level and survival rate. Four were lost to follow-up in the 172 patients. Through K–M single-factor analysis, the overall survival rate was closely correlated with FGF2 ($p < 0.001$), FGFBP1 ($p < 0.001$), FGFR3 expression ($p < 0.001$), lymph node metastasis ($p = 0.006$), vascular invasion ($p = 0.011$), nerve invasion ($p = 0.02$), and the treatment with postoperative chemoradiotherapy ($p = 0.002$) (Figures 3 and 4). However, there was no obvious relation, with sex, tumor size, degree of differentiation, and TNM. And progression-free survival is closely related to FGF2 ($p < 0.001$), FGFR3 ($p < 0.001$), and FGFBP1 ($p < 0.001$); lymph node metastasis ($p = 0.005$); vascular invasion ($p = 0.008$), and distant metastases ($p = 0.008$), as is shown in Figure 5 and Table 6. The progression-free survival of FGF2-, FGFR3-, and FGFBP1-positive patients was significantly lower than that of negative patients. Cox multivariate regression analysis showed that, as is shown in Table 6, vascular invasion ($p = 0.03$) and postoperative chemotherapy ($p = 0.001$) can significantly have an impact on the overall survival status of ESCC patients (Table 6). FGF2 ($p < 0.001$), FGFR3 ($p = 0.003$), vascular invasion ($p = 0.033$) and distant metastases ($p = 0.014$) can significantly have an influence on the progression-free survival status of patients (Table 6).

To sum up, we analyze the impact of various factors on the overall survival and progression-free survival. Patients with vascular metastases have a worse prognosis. Postoperative chemotherapy patients have a better prognosis than those who only underwent surgery. Patients with distant metastasis have a worse prognosis. The survival time of patients with lymph node metastasis was significantly lower than that of patients without lymph node metastasis. The overall survival of FGF2-, FGFR3-, and FGFBP1-positive patients is significantly lower than that of negative patients. For the patients with low expression of FGF2, FGFR3, and FGFBP1 are longer than that of patients with high expression in progression-free survival.

3.5. The Expression of FGF2, FGFR3, FGFBP1 Effect Overall Survival and Progression-Free Survival Time in Patients with Postoperative Chemotherapy. In our study, there are 51 patients (30.3%) underwent postoperative chemotherapy. 33 (64.7%) cases had high expression of FGF2, and 18 (35.3%) cases had negative expression. There were 32 positive cases of FGFR3 and 19 negative cases, with a positive rate of 62.7%. There were 30 cases (58.8%) who had high expression of FGFBP1 and 21 (41.2%) cases who had negative expression, as is shown in Table 7 and Figure 6. The results showed that patients with ESCC receiving postoperative chemoradiotherapy had longer survival time, but these patients with high expression of FGF2 ($p = 0.05$), FGFR3 ($p = 0.025$), and FGFBP1 ($p = 0.005$) had significantly lower overall survival than those with negative expression. In progression-free survival, patients with high expression of FGF2 ($p < 0.001$), FGFR3 ($p < 0.001$), and FGFBP1 ($p < 0.001$) had significantly lower survival than those with low expression.

Table 4: Correlation between FGF2 and FGFR3 and FGFBP1.

	FGF2		p	rs
	Positive	Negative		
FGFR3				
Positive	99 (57.5%)	12 (7%)	<0.001	0.612
Negative	18 (10.5%)	43 (25%)		
FGFBP1				
Positive	101 (58.7%)	11 (6.4%)	<0.001	0.649
Negative	16 (9.3%)	44 (25.6%)		

Table 5: Correlation between FGFR3 and FGFBP1.

	FGFR3		p	rs
	Positive	Negative		
FGFBP1				
Positive	98 (57%)	14 (8.1%)	<0.001	0.656
Negative	13 (7.6%)	47 (27.3%)		

4. Discussion

Fibroblast growth factor (FGF) represents 20 different proteins that are widely expressed in various tissues. Fibroblast growth factor (FGF) is involved in biological development and tissue homeostasis maintenance and is associated with angiogenesis and cancer progression [25]. FGF2 (basic FGF) is one of the most thoroughly studied members of the FGF family and has been shown to play a variety of biological roles in different cell and organ systems. It has also been shown to be involved in tumorigenesis and angiogenesis [13]. FGF2 in the tumor microenvironment plays a key role in regulating cancer stem-like cells (CSCs) [26], promoting the occurrence and development of tumors. In fibrosarcoma, cancer cells and their surrounding immune inflammatory cells overexpress or induce FGF2 expression, which plays a key role in tumor progression and angiogenesis [27]. In breast cancer cells, FGF2 showed high expression and was a

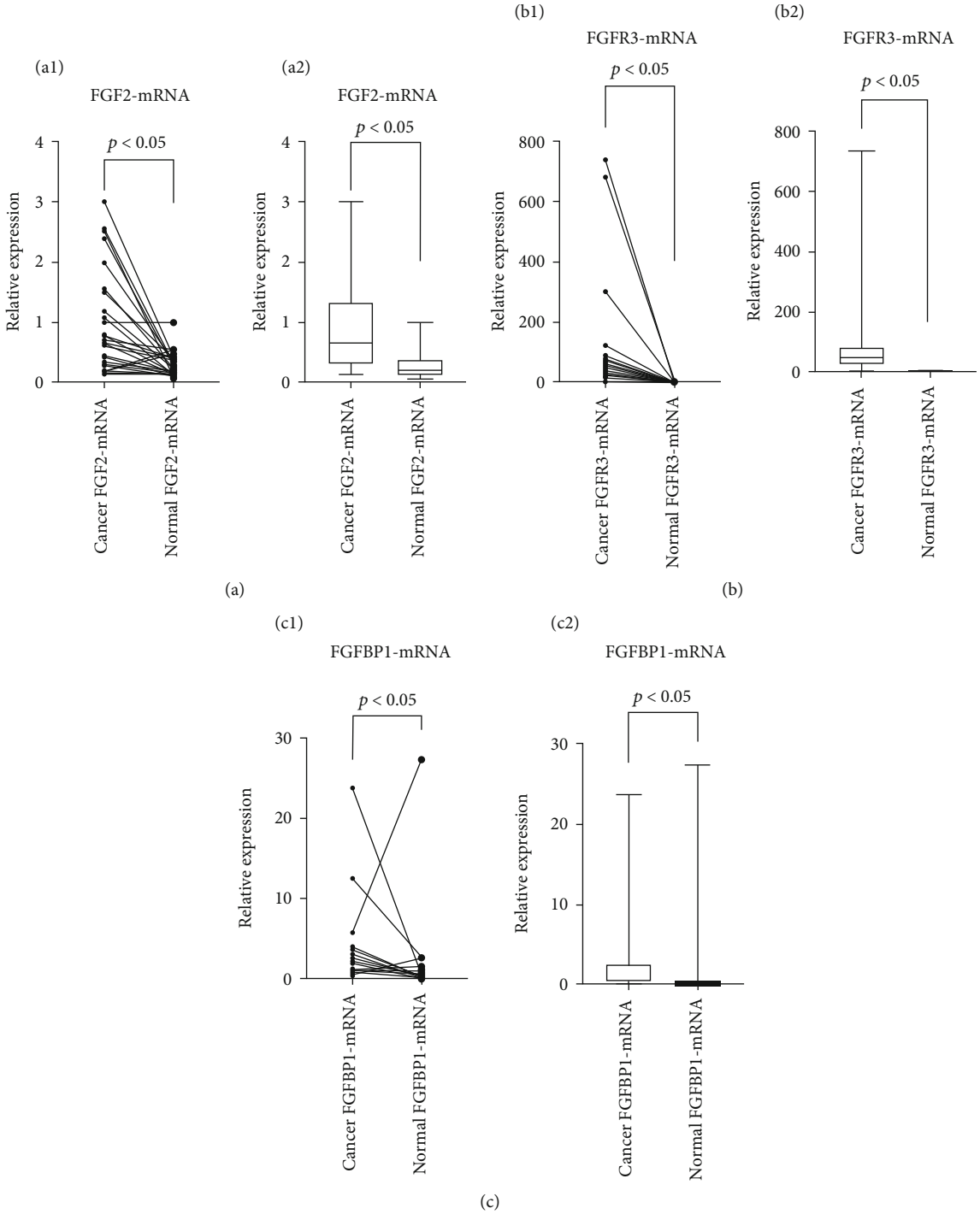


Figure 2: The expression of FGF2, FGFR3, and FGFBP1-mRNA in cancer tissues was significantly higher than that in adjacent tissues ($p < 0.05$).

powerful mitogen and an effective antiapoptotic substance, while inducing its invasion [28, 29]. Takase et al. analyzed the tissue specimens of 70 cases of esophageal squamous cell carcinoma by immunohistochemistry to investigate whether the expression level of FGF2 is related to the clinicopathological parameters of ESCC patients. According to the expression level of FGF2, they were further classified into weak positive group and strong positive group. The results showed that the strong positive group was positively correlated with the depth of infiltration, degree of vascular infiltration, and stage [30]. The results of our study showed that the expression of ESCC patients was significantly higher than that of normal esophageal tissues, and the positive rate was 68% (117/172), which was

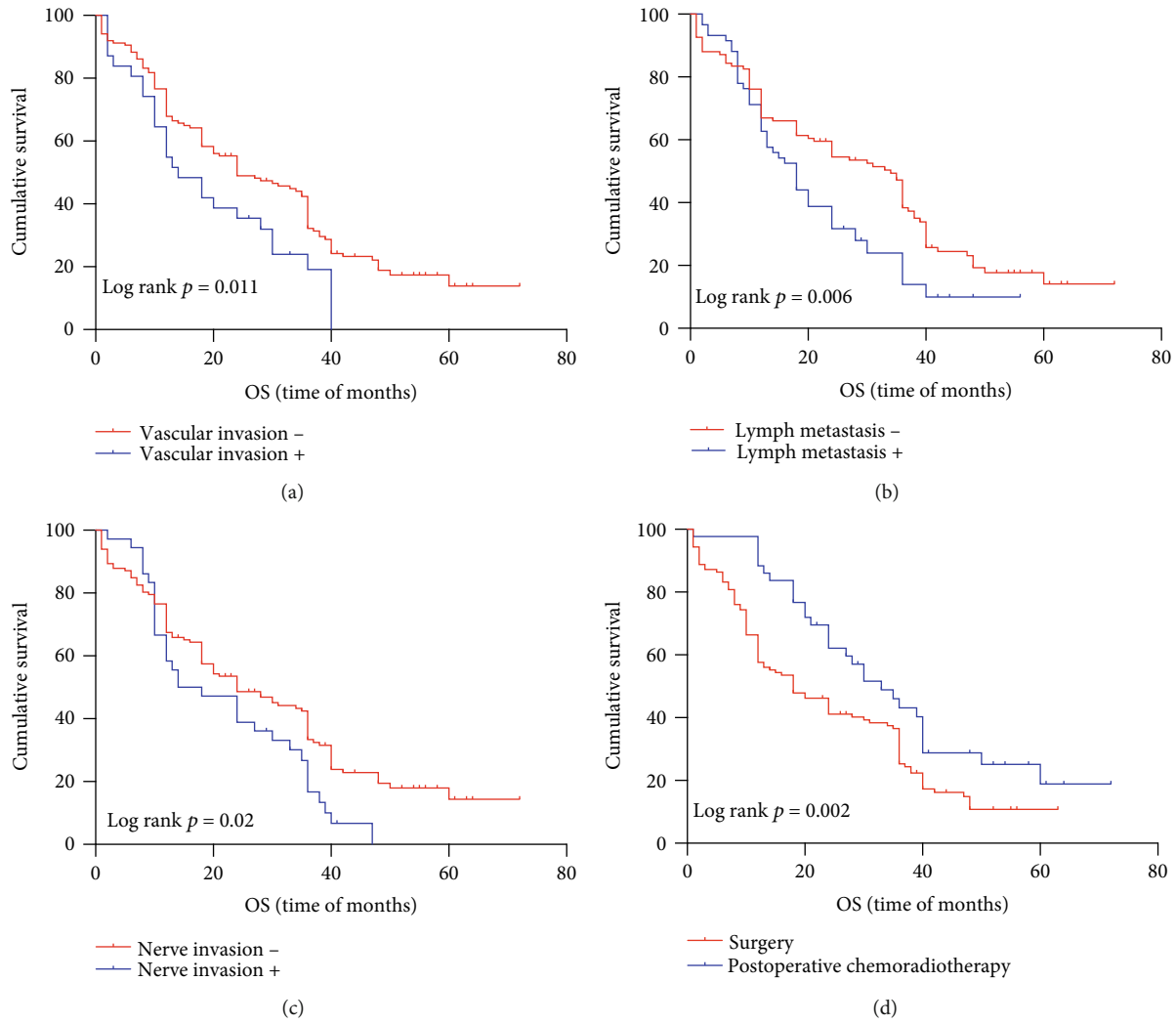


Figure 3: Kaplan–Meier curves for overall survival of ESCC with lymph node metastasis, vascular invasion, nerve invasion, and the treatment of surgery plus chemotherapy. (a) Patients with vascular invasion have a significantly shorter survival ($p = 0.011$); (b) patients with lymph node metastasis have a significantly shorter survival ($p = 0.006$); (c) patients with nerve invasion have a significantly shorter survival ($p = 0.02$); (d) patients who had postoperative chemoradiotherapy have a significantly longer survival ($p = 0.002$).

consistent with previous studies. Moreover, the expression of FGF2-mRNA in the cancer tissues was significantly higher than that in the adjacent tissues in RT-PCR test, which was consistent with the results of IHC. The high expression of FGF2 was related to the tumor size ($p = 0.026$), gender ($p = 0.047$), and lymph metastasis ($p = 0.007$), while the expression of FGF2 was not correlated with the race, age, tumor site, and pathological stage of ESCC cases and so on ($p > 0.05$). The expression of FGF2 is related to gender and may be related to the male-to-female ratio of patients. Therefore, it can be speculated that FGF2 may be a promoting effect on the tumor progression. After survival analysis, the prognosis of FGF2 positive was significantly worse. FGF2 expression is considered an independent prognostic factor affecting the progression-free survival ($p < 0.001$) ESCC patients by Cox multivariate regression analysis. These results suggest that FGF2 may be used as an independent prognostic indicator of progression-free survival in patients with esophageal squamous cell carcinoma.

FGFR3 germ line mutations cause fatal dysplasia, cartilage growth not congruent, and congenital disorders. FGFR3 somatic mutations or excessive FGFR3 protein expression can lead to the development of a variety of malignant tumors. FGFR3 excessive gene mutation and protein expression were first discovered in bladder tumor. Besides, FGFR3 overexpression was found in gastric cancer and liver cancer. Studies have shown that dysfunction in FGFR3 or mutations of FGFR3 are highly associated with multiple cancers, such as multiple myeloma, bladder cancer [31], breast cancer [32], and colorectal cancer [33]. The activation of the FGFR3 signaling pathway can promote tumor growth, metastasis, and drug resistance [34, 35]. Studies have shown that that FGFR3 expression promoted tumor cell proliferation immunohistochemical analysis of early esophageal squamous cell carcinoma [36]. In previous studies of esophageal cancer,

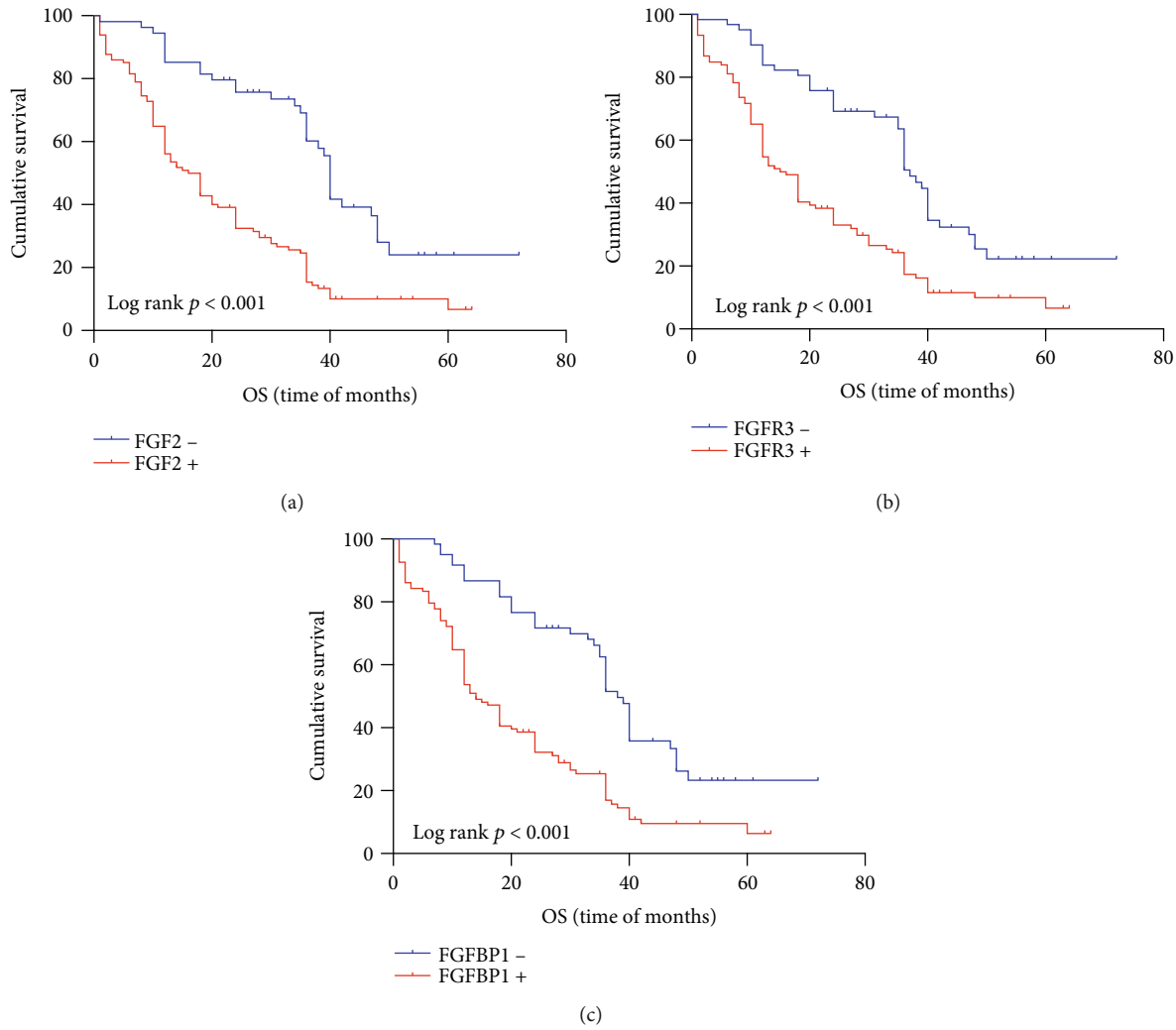


Figure 4: Kaplan–Meier curves for overall survival of ESCC with FGF2, FGFR3, and FGFBP1 expression. (a) Patients expressing high level of FGF2 have a significantly shorter survival ($p < 0.001$); (b) patients expressing high level of FGFR3 have a significantly shorter survival ($p < 0.001$); (c) patients expressing high level of FGFBP1 have a significantly shorter survival ($p < 0.001$).

FGFR3 was associated with tumor proliferation. In this study, the expression of FGFR3 in ESCC patients was significantly higher than that in tissues adjacent to carcinoma (64.5% (111/172)), which was consistent with FGFR3 expression in gastric and liver cancers in previous studies. And in RT-PCR, FGFR3-mRNA expression in cancer tissues was significantly higher than that in adjacent tissues ($p < 0.001$). Furthermore, the expression of FGFR3 in ESCC carcinoma was significantly higher than that in surrounding normal tissues. In this study, FGFR3 expression was associated with tumor differentiation ($p = 0.043$ and $p < 0.05$), lymph node metastasis ($p = 0.078$ and $p < 0.1$), and race ($p = 0.033$ and $p < 0.05$), suggesting that FGFR3 may have an influence on the tumor development. FGFR3 expression is considered an independent prognostic factor affecting the overall survival ($p < 0.001$) and the progression-free survival ($p = 0.003$) in patients with ESCC by Cox multivariate regression analysis. The survival analysis showed that the FGFR3-positive patients had a poor prognosis. In this study, only advanced esophageal squamous cell carcinoma was selected, because of the greater influence of early and advanced cancers on the prognosis. The previous studies have suggested that FGFR3 may be of diagnostic value in early carcinoma, and further study of FGFR3 expression in early carcinoma of esophageal squamous cell carcinoma is warranted.

In the normal adult tissues, some studies have shown that FGFBP1 has also been shown to induce tumorigenic potential in epithelial cells [37] and to be highly expressed in oral cancer cell lines and tissues [38]. And other studies have found that overexpression of FGFBP1 can lead to skin diseases, such as psoriasis, actinic keratosis, and squamous cell carcinoma of the skin [39]. The previous studies have found that ed-71 (an anticancer agent for squamous cell carcinoma) inhibits tumor growth by inhibiting tumor angiogenesis in squamous cell carcinoma of the skin. One of the functions of ed-71 is to regulate the expression of HBp17/fgfbp-1 in tumors, which can affect the release of fgf-2 by ECM and

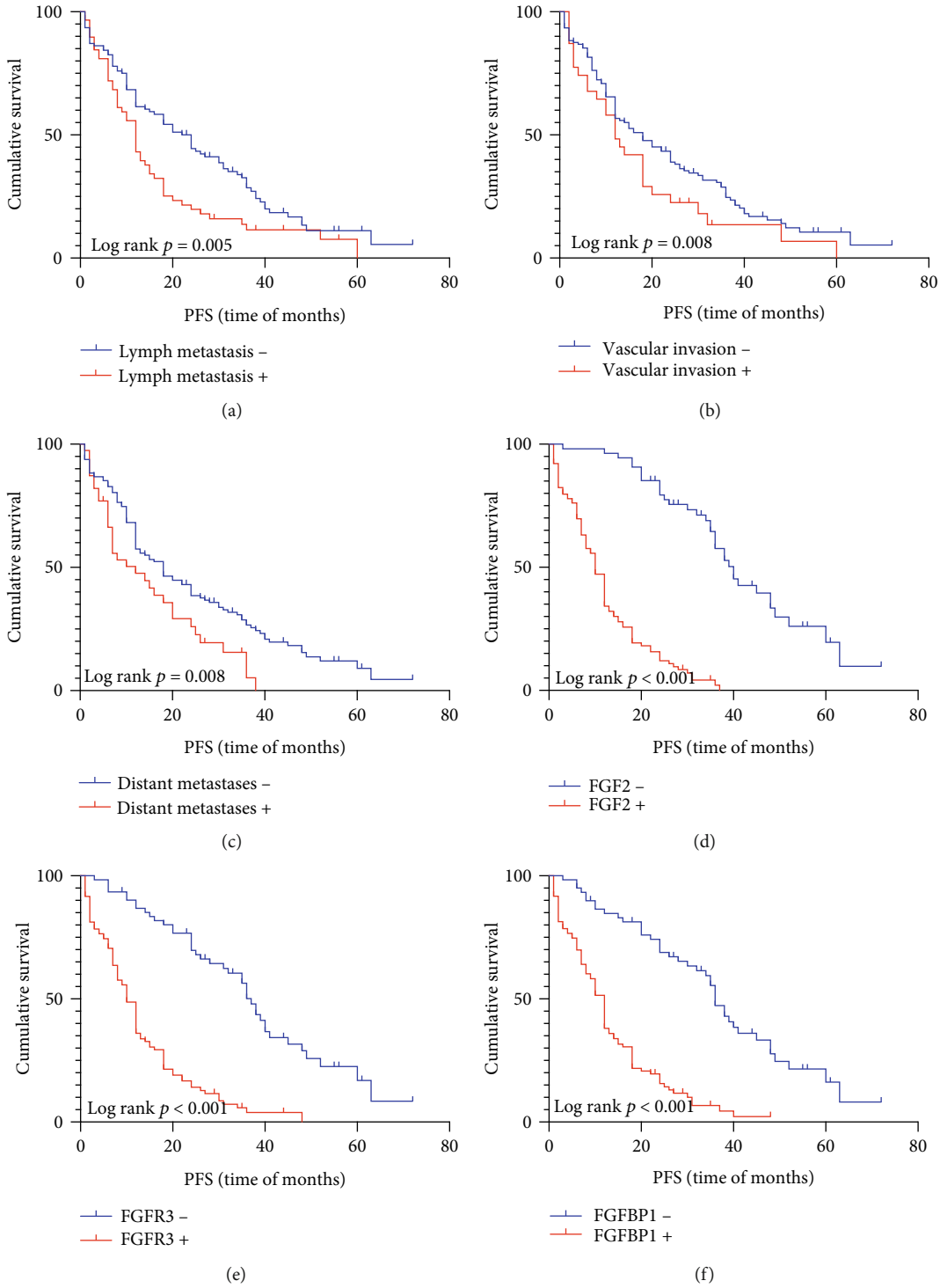


FIGURE 5: Kaplan–Meier curves for PFS of ESCC. (a) Patients with lymph node metastasis have a significantly shorter survival ($p = 0.005$); (b) patients with vascular invasion have a significantly shorter survival ($p = 0.008$); (c) the prognosis of patients with distant metastasis is poor ($p = 0.008$); (d) patients expressing high level of FGF2 have a significantly shorter survival ($p < 0.001$); (e) patients expressing high level of FGFR3 have a significantly shorter survival ($p < 0.001$); (f) patients expressing high level of FGFBP1 have a significantly shorter survival ($p < 0.001$).

angiogenesis [40, 41]. However, the expression in ESCC is unclear. The expression of this research shows that FGFBP1 from patients with esophageal is a high expression (70.2%), thus making the results consistent with the expression of colon cancer, pancreatic cancer, and breast cancer. Moreover, RT-PCR further confirmed that the expression of FGFBP1 in ESCC tissues was higher than that in normal tissues ($p = 0.001$). The expression of FGFBP1 with ESCC

Table 6: Univariable and multivariable analyses for overall survival and progression-free survival.

Variable	Overall survival				Progression-free survival			
	Univariable		Multivariable		Univariable		Multivariable	
	Median	*p* value	HR (95% CI)	*p* value	Median	*p* value	HR (95% CI)	*p* value
Gender								
Male	24	0.286			15	0.127		
Female	36				18			
Race								
Han	24	0.604			18	0.515		
Kazakh	24				13			
Age (years)								
<65	28	0.101			18	0.292		
≥65	15				12			
Tumor size								
<3 cm	36	0.203			18	0.839		
≥3 cm	20				15			
Tumor site								
Upper	36				48			
Middle	20	0.31			12	0.178		
Lower	24				18			
Differentiation								
Well	30				18			
Moderate	21	0.422			14	0.158		
Poor	24				20			
pTNM		0.065				0.252		
IB	34				36			
IIA.B	24				16			
IIIA.B,C	20				14			
Lymph metastasis								
Negative	34	0.006	0.75 (0.51-1.09)	0.131	24	0.005	0.912 (0.631-1.319)	0.626
Positive	18				12			
Vascular invasion								
Negative	24	0.011	0.60 (0.38-0.95)	0.03	18	0.008	0.616 (0.394-0.963)	0.033
Positive	14				12			
Nerve invasion								
Negative	24	0.02	0.76 (0.49-1.16)	0.204	15	0.369		
Positive	14				16			
Distant metastases								
Negative	21	0.934			18	0.008	0.597 (0.395-0.903)	0.014
Positive	24				12			
Treatment								
Surgery	15	0.002	0.54(0.36-0.76)	0.001	13	0.259		
Postoperative chemoradiotherapy	33				20			
FGF2								
Negative	40	<0.001	0.57 (0.32-1.01)	0.056	40	<0.001	0.183 (0.097-0.364)	<0.001
Positive	16				10			
FGFR3								
Negative	37	<0.001	0.69 (0.42-1.15)	0.157	37	<0.001	0.465 (0.281-0.769)	0.003
Positive	15				10			

Table 6: Continued.

Variable	Overall survival				Progression-free survival			
	Univariable		Multivariable		Univariable		Multivariable	
	Median	p value	HR (95% CI)	p value	Median	p value	HR (95% CI)	p value
FGFBP1								
Negative	38	<0.001	0.84 (0.48-1.49)	0.541	36	<0.001	0.870 (0.477-1.587)	0.65
Positive	14				12			

Table 7: Univariate analysis of overall and progression-free survival in patients with ESCC postoperative chemoradiotherapy.

Variable		Overall survival			Progression-free survival		
		Median	Log rank (chi-square)	p	Median	Log rank (chi-square)	p
FGFR3	Negative	40	3.691	0.05	36	12.154	<0.001
	Positive	28			16		
FGF2	Negative	40	5.047	0.025	36	20.163	<0.001
	Positive	27			16		
FGFBP1	Negative	40	7.901	0.005	36	20.403	<0.001
	Positive	24			12		

cases of tumor differentiation ($p = 0.012$), age ($p = 0.045$), and lymph node metastasis ($p = 0.032$) has more obvious relationship, rather than gender, tumor size, tumor location, pathological stage, and vascular invasion of clinical pathology features. We can speculate that FGFBP1 may promote the development of esophageal squamous cell carcinoma through FGF2. Through survival analysis, there was a significant difference in survival time between FGFBP1-positive patients and negative patients. Patients with high expression of FGFBP1 had worse prognosis.

In the previous studies, the FGF/FGFR system is a key factor in tumor-microenvironment interactions [42, 43]. We found that fibroblast growth factor-binding protein (FGFBP1) was the carrier molecule of FGF2, which was first found in tumor cell lines [44]. Subsequent studies have shown that FGFBP1 binds to the FGF2 released from HSPG and then transports the bound FGF2 to the target cell surface [45, 46] to bind to FGFR. FGF/FGFR3 axis may induce carcinogenic effects by promoting cancer progression and increasing angiogenesis potential, leading to metastatic tumor phenotypes (Figure 7). Blocking one or more components of the FGFR signal pathway is being examined in preclinical studies and some clinical trials. However, early findings revealed that alterations in the FGFR gene do not occur uniformly across the various types/subtypes of cancer, suggesting the existence of complex interactions that vary between cancer types/subtypes [47, 48]. The results of our study showed a positive correlation among these factors, suggesting that the FGF2-FGFR3 axis may play a certain role in promoting the occurrence of ESCC and affecting the prognosis of ESCC.

The studies have shown that FGF2 is frequently dysregulated in cancer, especially in advanced stages of disease. The upregulation of FGF2 or FGFRs can promote resistance to chemotherapy. FGF2 is currently being evaluated in clinical studies as a potential predictive biomarker for hematological and solid tumors. FGF2/FGFR inhibitors are being developed and evaluated as monotherapy or as part of a combination therapy for the treatment of different types of cancer [49]. The finding was also found in our study that the survival times of patients with negative expression of FGF2, FGFR3, and FGFBP1 were significantly higher than that of patients with positive expression in 51 patients who received postoperative chemotherapy. It is concluded that the high expression of FGF2, FGFR3, and FGFBP1 in patients with ESCC may be prone to be resistant to chemotherapeutic drugs or radiotherapy. This is consistent with the relevant reports. Therefore, the inhibition of FGF2, FGFR3, and FGFBP1 may enhance the efficacy of chemotherapy, which is hopeful to make it an irreplaceable sensitizing target for cancer treatment. In this study, only immunohistochemistry and PCR were used. Further verification should be done by cell or animal tests.

In summary, the high expression of FGF2 was related to the tumor size of ESCC tissues and lymph node metastasis; the FGFR3 expression was associated with tumor differentiation, race, and lymph node metastasis. The expression of FGFBP1 with ESCC was associated with tumor differentiation degree, age, and lymph node metastasis. The protein and mRNA expressions of FGF2, FGFR3, and FGFBP1 were higher in the ESCC than in the adjacent tissues. FGF2, FGFBP1, and FGFR3 can promote the ESCC progression. FGF2 was significantly correlated with FGFR3 and FGFBP1, and FGFR3 was correlated with FGFBP1. The study further confirmed that the FGF2-FGFR3 axis may promote the progression of esophageal squamous cell

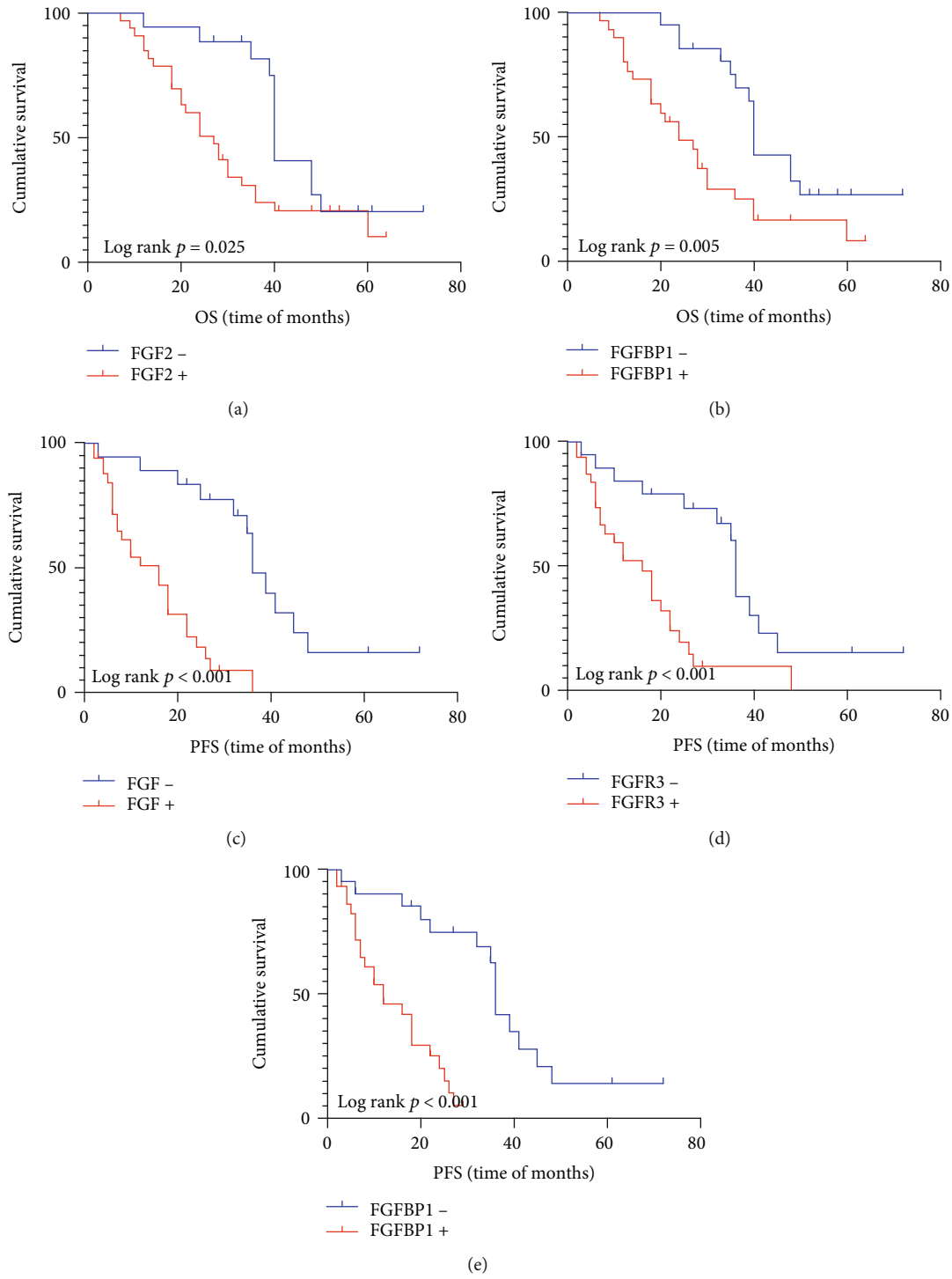


Figure 6: Kaplan–Meier survival analysis: total and progression-free survival in patients undergoing postoperative chemotherapy. (a) The OS of patients with FGF2 overexpression was shorter in ESCC ($p = 0.025$); (b) the high expression of FGFR3 had a poor OS in ESCC ($p = 0.05$); (c) the high expression of FGFBP1 had a poor OS in ESCC ($p = 0.005$); (c) the PFS of patients with FGF2 overexpression was shorter in ESCC ($p < 0.001$); (e) the high expression of FGFR3 had a poor PFS in ESCC ($p < 0.001$); (f) the high expression of FGFBP1 had a poor PFS in ESCC ($p < 0.001$).

carcinoma. The FGF2-FGFR3 axis may be a new direction of targeted therapy for esophageal squamous cell carcinoma. Furthermore, high expression of FGF2, FGFR3, and FGFBP1 may increase drug resistance and reduce survival. Therefore, blocking the FGF2-FGFR3 axis may inhibit the development of tumors. The inhibition of FGF2, FGFR3, and FGFBP1 may be further increased susceptibility to other chemotherapy drugs. The results of

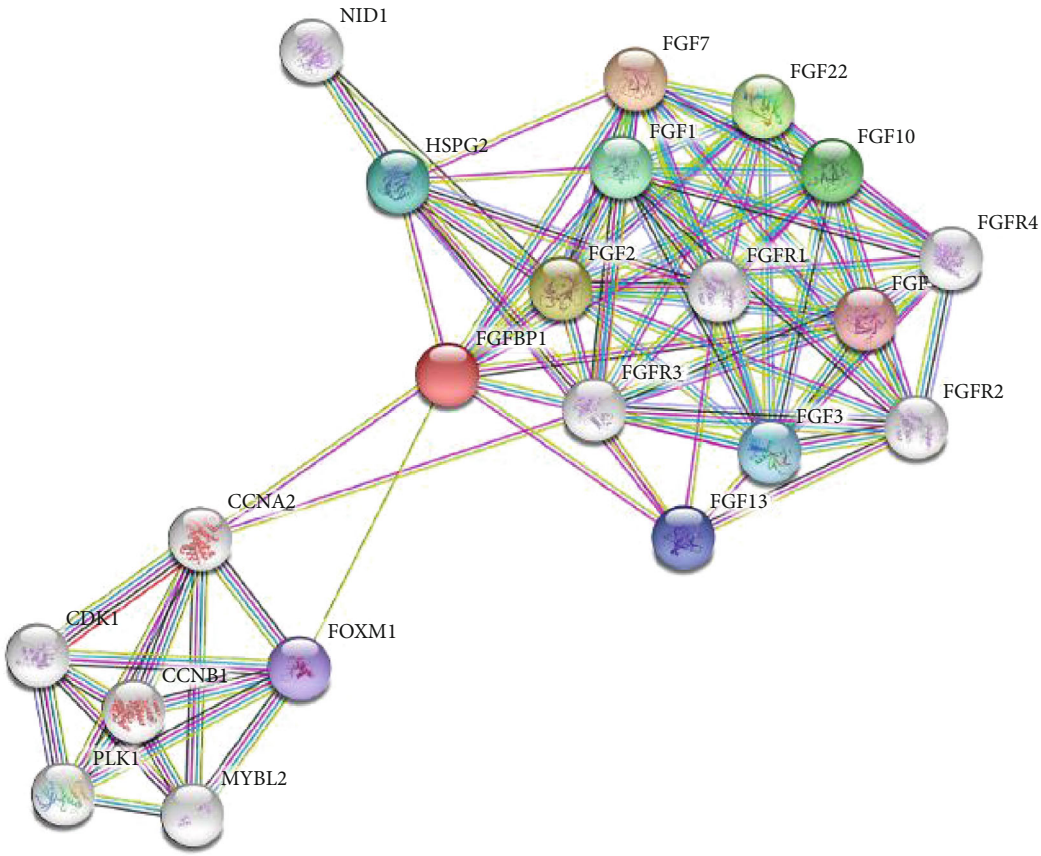


Figure 7: The connection diagram of FGF2, FGFR3, and FGFBP1.

multivariate analysis showed that both FGF2 and FGFR3 affected prognosis. Therefore, FGF2 and FGFR3 may be used as molecular markers for prognosis of ESCC.

Data Availability

The data of immunohistochemical and PCR used to support the findings of this study are included within the supplementary information file(s). The data used to support the findings of this study were supplied by Yuqing Ma under license. Because it involves patient privacy, so cannot be made freely available. Requests for access to these data should be made to Yuqing Ma (yuqingm0928@126.com).

Ethical Approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

Consent

Signed written informed consent was obtained from all participants before the study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YQM, WJZ, and MYL designed the study and performed the experiments. WJZ, CL, YXZ, WYL, and SSX collected the data. And SSX and ZWJ analyzed the data. WJZ wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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Supplementary Materials

Table 1(s)-Table 3(s): the data of immunohistochemical patients. Table 4s-7s: mPCR data information. *(Supplementary materials)*

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Research Article

Immunosuppressive Phenotype of Esophagus Tumors Stroma

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Background. Tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs) contribute significantly to the development of immunosuppressive properties of a tumor. In this study, we performed immunohistochemical analysis of immune cells of esophageal tumors stroma. *Methods*. Paraffin-embedded tissue specimens from 48 esophageal squamous cell carcinoma (ESCC) patients were retrospectively collected for immunohistochemical analysis of stromal cells. For staining of macrophages, CD68, CD163, CD206, PU.1, and iNOS were used. For T cell detection, CD8, CD3, and FOXP3 were used. Also, we performed staining for PD-L1 that can be expressed on TAMs and tumor cells. Clinicopathological and survival data were collected and analyzed using the χ^2 and Fisher exact tests, Kaplan–Meier curves, and the log-rank test. The correlation analysis was performed with Spearman's rank correlation coefficient. *Results*. We found that FOXP3 expression was associated with age ($p = 0.042$) and iNOS expression was associated with the disease stage ($p = 0.044$). In addition, FOXP3 and CD163 appeared to be markers of good prognosis (HR = 0.4420, $p = 0.0325$, and HR = 0.4447, $p = 0.0456$, respectively). Significant association between PU.1+ and CD68+ macrophages ($r = 0.833$; $p \leq 0.001$) and between PU.1+ and CD163+ macrophages ($r = 0.500$; $p \leq 0.001$) was established; positive association between PU.1 and CD206 expression was also observed ($r = 0.250$; $p = 0.043$). *Conclusions*. Large amounts of CD163+ macrophages and FOXP3+ T cells appear to be markers of good prognosis of ESCC. The number of PU.1+ macrophages strongly correlates with the number of CD68+ macrophages; therefore, usage of PU.1 as a potential macrophage marker can be recommended for esophageal tumors.

1. Introduction

Esophageal cancer is the sixth frequent cause of death among malignant tumors. Due to late-stage diagnosis, about 70% of patients die within 1 year after diagnosis. There are two main subtypes of esophageal cancer described: esophageal squamous cell carcinoma (ESCC) comprising 90% esophageal cancer cases and esophageal adenocarcinoma (EAC).

Available data indicate that in order to understand the pathogenesis of esophageal cancer, it is necessary to understand not only the molecular repertoire of the tumor cells but also the properties of the cells of the tumor microenvironment, which contains various cells of the immune system that support the development of the tumor at all its stages. The escape of the tumor from immunological control is crucial for the survival, progression, and metastasis of the tumor. Tumor cells can suppress the antitumor immune response through the production of various soluble factors, which, in turn, attract and direct the differentiation and activation of stromal cells in the direction necessary for the tumor. In this work, we examined cell populations associated with the tumor immune escape, namely, macrophages and T cells.

TAMs show a number of protumorigenic features. It is widely accepted that macrophages may display a broad spectrum of phenotypes where type 1 (M1) and type 2 (M2) macrophages represent its extremes. M1 stimulate inflammation, produce proinflammatory cytokines, and show antitumor cytotoxic activity; M2 produce anti-inflammatory cytokines, extracellular matrix components, and remodeling enzymes and show high phagocytic and low cytotoxic activities [1–3]. TAMs support tumor progression by producing proangiogenic and growth factors. They are also thought to inhibit

T cell effector functions by releasing immunosuppressive cytokines [3, 4]. In most of studied cancers, the presence of increased number of TAMs appears to be a marker of poor prognosis. This is also the case for esophageal cancer [5].

Tumor-infiltrating lymphocytes (TILs) represent another important part of tumor stromal cells. They are found in different tumors, and their population is mainly comprised of CD3+ and CD8+ T cells. CD3+ T cells have antitumor activity [6]. As TAMs, CD3+ TILs show both antitumor and tumor-supporting activities. In contrast, CD8+ T lymphocytes have cytotoxic activity against cancer cells, and these T cells could play an important role in antitumor immunity. Regulatory T cells (Tregs) also show immunosuppressive activity in cancer. In a healthy organism, Tregs control activation and expansion of B and T cells, as well as NK cell cytotoxicity; however, in cancer, they inhibit antitumor immune responses [7]. Interestingly, Tregs may act differently at different stages of tumor development. At the initial stages, Tregs suppress inflammation that may lead to carcinogenesis but later diminish antitumor immunity via the secretion of immunosuppressive cytokines and inhibition of cytotoxic cell function [8].

Recent advances in cancer immunological therapeutics have revealed the importance of programmed death-1-(PD-1-) activated signaling. The combination of PD-1 and its ligand PD-L1 is the key immune checkpoint for inhibition of T cell activation. Recently developed PD-L1 inhibitor antibodies are now used for treatment of various cancers including esophageal cancer. However, in contrast to many other tumors, the association of PD-L1 expression with the clinicopathological relationship in ESCC remains controversial. Some studies demonstrated that PD-L1 expression correlates with poor prognosis [9], while others suggested that PD-L1 could be a favorable prognostic indicator in ESCC [10].

In this study, we have examined the prognostic impact of different components of tumor stroma basing on immunohistochemical analysis of macrophage and T cell markers in a group of 48 curatively resected esophageal squamous cancers. We established that out of all macrophage markers studied, only CD206 correlates with the clinicopathological features of the tumor. Analysis of survival revealed that the number of CD163+ TAMs and FOXP3+ TILs correlates with prolonged survival of the patients. We also tested PU.1 as a potential general marker for macrophages and demonstrated its high correlation with CD68, which confirms our hypothesis of the possible use of nuclear PU.1 staining for labelling TAMs.

2. Materials and Methods

2.1. Study Population. A total of 48 surgically resected and formalin-fixed paraffin-embedded (FFPE) human ESCC tissues were collected from the Clinical Oncology Department of N. N. Blokhin Russian Cancer Research Centre (Moscow, Russia) (collected from 2005 to 2012). The patients consisted of 36 men and 12 women with an age range of 43–79 years old and mean age of 61 years old; all had been diagnosed with ESCC. All specimens were sectioned into 5 μm sections and subjected to conventional hematoxylin and eosin staining.

Table 1: Study population.

Category	All cases
Age	
≤60	26 (54%)
>60	22 (46%)
Gender	
Male	36 (75%)
Female	12 (25%)
Stage	
I-II	25 (52%)
III-IV	23 (48%)
Nodal Status	
N-	25 (52%)
N+	23 (48%)
Histologic grade	
G1/2	38 (79%)
G3	10 (21%)

A diagnosis of ESCC was confirmed by a pathologist following the World Health Organization histological tumor classification criteria. There were 10 cases of well-differentiated ESCC, 28 cases of moderately differentiated ESCC, and 10 cases of poorly differentiated ESCC. There were 23 cases with lymph node metastasis, 25 cases in clinical stages I–II, and 23 cases in clinical stages III–IV (Table 1). The survival status of all patients was followed up by postcontact until December 2019. The median follow-up for living patients was 40 months (range, 2–152 months). Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last follow-up for surviving patients. Among the 37 patients who were recruited, 23 (62.0%) died, and 14 (38.0%) remained alive during the follow-up period.

The Institutional Review Board of N.N. Blokhin Russian Cancer Research Center approved the project (approval date 09/2018), and all patients, who were involved in the study, gave written informed consents that their samples could be used for research purposes. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Data were analyzed anonymously. All potential participants who declined to participate or otherwise did not participate were eligible for treatment (if applicable) and were not disadvantaged in any other way by not participating in the study.

2.2. Immunohistochemical Analysis. Standard immunohistochemical procedure was used for staining FFPE sections with antibodies against stromal cell markers. We used the following antibodies: mouse anti-CD163 (Clone 10D6; BIOCARE, USA, 1 : 100 dilution), rabbit anti-CD206 (HPA004114; Sigma, USA, 1 : 2000 dilution), rabbit anti-iNOS (SAB5500152; Sigma, USA, 1 : 150 dilution), rabbit anti-FOXP3 (Clone D2W8E; Cell Signaling Technology, USA, 1 : 200 dilution), rabbit PU.1 (Clone 9G7; Cell Signaling Technology, USA, 1 : 200 dilution), rabbit anti-PD-L1 (E1L3N; Cell Signaling Technology, USA, 1 : 200 dilution), rabbit anti-CD68 (Clone GR021, 61-0184 Genemed, USA, 1 : 100 dilution), mouse

Table 2: Clinicopathological characteristics and TAM markers in ESCC.

	CD68			CD163			CD206			PU.1			iNOS		
	High	Low	p	High	Low	p	High	Low	p	High	Low	p	High	Low	p
Stage															
I-II	12	13	>0.999	13	12	>0.999	13	12	>0.999	13	12	>0.999	1	24	0.044*
III-IV	12	11		11	12		11	12		11	12		6	17	
Nodal status															
N-	11	14	0.564	13	12	>0.999	13	12	>0.999	12	13	>0.999	2	23	0.237
N+	13	10		11	12		11	12		12	11		5	18	
Histological grade															
G1/2	19	19	>0.999	19	19	>0.999	22	16	0.072	19	19	>0.999	6	32	>0.999
G3	5	5		5	5		2	8		5	5		1	9	
Age															
≤60	13	13	>0.999	11	15	0.385	12	14	0.773	12	14	0.773	6	20	0.106
>60	11	11		13	9		12	10		12	10		1	21	
Gender															
Male	18	18	>0.999	18	18	>0.999	16	20	0.318	18	18	>0.999	6	30	0.662
Female	6	6		6	6		8	4		6	6		1	11	

*Statistically significant.

anti-CD8 (Clone CD8/144B, 61-0124 Genemed, USA, 1 : 100 dilution), and rabbit anti-CD3 (61-0011 Genemed, USA, 1 : 100 dilution). We used UltraVision Quanto Detection System HRP DAB (Thermo Fisher Scientific, USA).

2.3. IHC Scoring. IHC scoring was done as described [11–14] with modifications. Macrophages and T cells were counted in tumor islets and in stroma. The numbers of CD68-, CD163-, CD206-, CD3-, CD8-, FOXP3-, and PU.1-positive cells in immunohistochemical staining were counted in 10 independent high-power microscopic fields (400x) of tumor tissue. The mean of 10 values was calculated and expressed as mean (standard deviation). Patients were divided into two groups according to the median values (low expression less or equal to than median value and high expression more than median value). These subgroups were used for further analysis. FOXP3 and PU.1 are transcription factors; therefore, only cells showing nuclear staining were counted.

For iNOS, the sample was considered to have low expression if less than 1% of tumor cells showed positive staining. Samples having more than 1% tumor cells expressing iNOS were considered high expressing.

For evaluation of PD-L1 expression, we used Combined Positive Score (CPS) (PD-L1 IHC 22C3 pharmDx Interpretation Manual—Esophageal Squamous Cell Carcinoma), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells, multiplied by 100. According to obtained results, the samples were divided into low expression (less than 1%) and high expression (more than 1%) groups.

2.4. Statistical Analysis. The statistical analysis was performed with GraphPad Prism, Version 8.3, software (San Diego, CA, USA). χ^2 and Fisher exact tests (for categorical variables) were used to compare the differences between the expression of CD68 and others and clinicopathological parameters of ESCCs. Spearman's rank correlation method was used to evaluate the correlations between the amounts of different inflammatory cell types in tumor stroma. Survival length was determined from the date of surgery to death or the date of the last clinical attendance. Survival curves were derived using the Kaplan–Meier method, and differences between curves were analyzed using the log-rank test. In all analyses, p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. TAM Phenotype. We used CD68 as a common macrophage marker, CD163 and CD206 as M2 markers, and iNOS as an M1 marker. As an additional general macrophage marker, we used PU.1. We selected PU.1 due to its relatively specific macrophage expression and nuclear pattern of staining which generally simplifies the scoring; in contrast, CD68, CD163, and CD206 demonstrate diffuse membrane and cytoplasmic staining that may lead to uncertainties in quantification.

We found CD68+, CD163+, and CD206+ TAMs distributed in both tumor stroma and tumor islets. By immunohistochemical analysis, in tumor tissue, the median level of CD68+ cells/HPF was 49 (range, 25–87), the median level of CD163+ cells/HPF was 45 (range, 9–104), the median level of CD206+ cells/HPF was 7 (range, 3–37), and the median level of PU.1+ cells/HPF was 58 (range, 12–115). Analyzed cases were divided into groups with high and low number of M2 macrophages as described above. These groups were used to analyze the association with clinicopathological characteristics. For CD206, a tendency for correlation with the histologic grade was observed, though it was not statistically significant ($p = 0.072$). No significant correlations were found between CD68+, CD163+, or PU.1+ and clinical characteristics ($p > 0.05$; Table 2).

Table 3: Clinicopathological characteristics and TIL markers in ESCC.

	CD3			CD8			FOXP3		
	High	Low	p	High	Low	p	High	Low	p
Stage									
I-II	15	10	0.248	15	10	0.248	12	13	>0.999
III-IV	9	14		9	14		12	11	
Nodal status									
N-	15	10	0.248	15	10	0.248	14	11	0.564
N+	9	14		9	14		10	13	
Histological grade									
G1/2	19	19	>0.999	20	18	0.724	19	19	>0.999
G3	5	5		4	6		5	5	
Age									
≤60	12	14	0.776	11	15	0.385	9	17	0.042*
>60	12	10		13	9		15	7	
Gender									
Male	18	18	>0.999	17	19	0.740	15	21	0.093
Female	6	6		7	5		9	3	

*Statistically significant.

Further, we demonstrate that none of the samples contained iNOS+ type 1 macrophages. Though iNOS expression in tumor cells was detected in 12 out of 48 samples in 5 cases, this expression was in less than 1% of cells. Expression of iNOS correlated with the disease stage ($p = 0.044$).

3.2. Tumor-Infiltrating Lymphocyte Phenotype. For tumor-infiltrating T cells, the situation was similar to that with macrophage. In nearly all samples (98%), CD3+ cells were detected within tumor stroma and tumor islets, and CD8+ cells were detected in 96% of cases. By immunohistochemical analysis, in tumor tissue, the median level of CD3+ cells/HPF was 40 (range, 16–126), CD8+ cells/HPF was 22 (range 5-65), and FOXP3+ cells/HPF was 5 (range 0-46). For the analysis of correlation with clinical data, the same approach as for macrophages was used. Samples were divided into high- and low-density TIL groups according to positive cell count and evaluated possible correlations with clinicopathological parameters, including age, gender, histological grade, nodal status, and clinical stages (Table 3).

Statistically significant correlation was found solely for FOXP3+ that correlated with the age of patients ($p = 0.042$).

Programmed death ligand 1 (PD-L1) is a ligand for the inhibitory programmed cell death protein 1 (PD-1), which is targeted by several anti-PD-1 and PD-L1 drugs for a variety of human cancers including metastatic squamous cell carcinoma of the esophagus. In our study group, 27% of samples were PD-L1-negative and 62.5% with Combined Positive Score lower than 1% (including PD-L1 negative samples). No statistically significant correlations of PD-L1 expression and clinical parameters were found (data not shown).

3.3. Survival Analysis. To identify markers of potential prognostic significance in the patients with ESCC, the impacts of TAMs and TIL subgroup and other clinicopathological parameters on the prognosis were explored. To establish the prognostic effect of these clinicopathologic characteristics and markers of immune cells, univariate analysis was used (results are presented in Table 4).

Table 4: Statistical analysis of the prognostic value of immune cells of tumor stroma.

	Univariate analysis		
	HR	95% CI	p
CD3 (high/low)	0.6930	(0.2996-1.603)	0.3623
CD8 (high/low)	0.8064	(0.3478-1.869)	0.5979
FOXP3 (high/low)	0.4420	(0.1985-0.9842)	0.0325*
CD68 (high/low)	0.8953	(0.4038-1.985)	0.7781
CD163 (high/low)	0.4447	(0.1957-1.010)	0.0456*
CD206 (high/low)	0.9158	(0.3994-2.100)	0.8292
iNOS (high/low)	0.6928	(0.2635-1.821)	0.4929
PU.1 (high/low)	0.6414	(0.2796-1.472)	0.2700
PD-L1 (high/low)	0.7251	(0.3171-1.658)	0.4504

*Statistically significant.

We established that increased CD163+ macrophages and FOXP3+ lymphocytes were significantly associated with prolonged overall survival (OS) in ESCC ($p = 0.0456$ and $p = 0.0325$, respectively), and the Kaplan–Meier figures are shown in Figure 1. For none of the other markers, statistically significant correlation was found.

3.4. PU.1 Is a New General Macrophage Marker. There is an urgent need for new macrophage markers suitable for immunohistochemical analysis showing nuclear staining. As such a marker, we used PU.1 in this study. It has a nuclear pattern of expression, which makes it easier to evaluate the data and

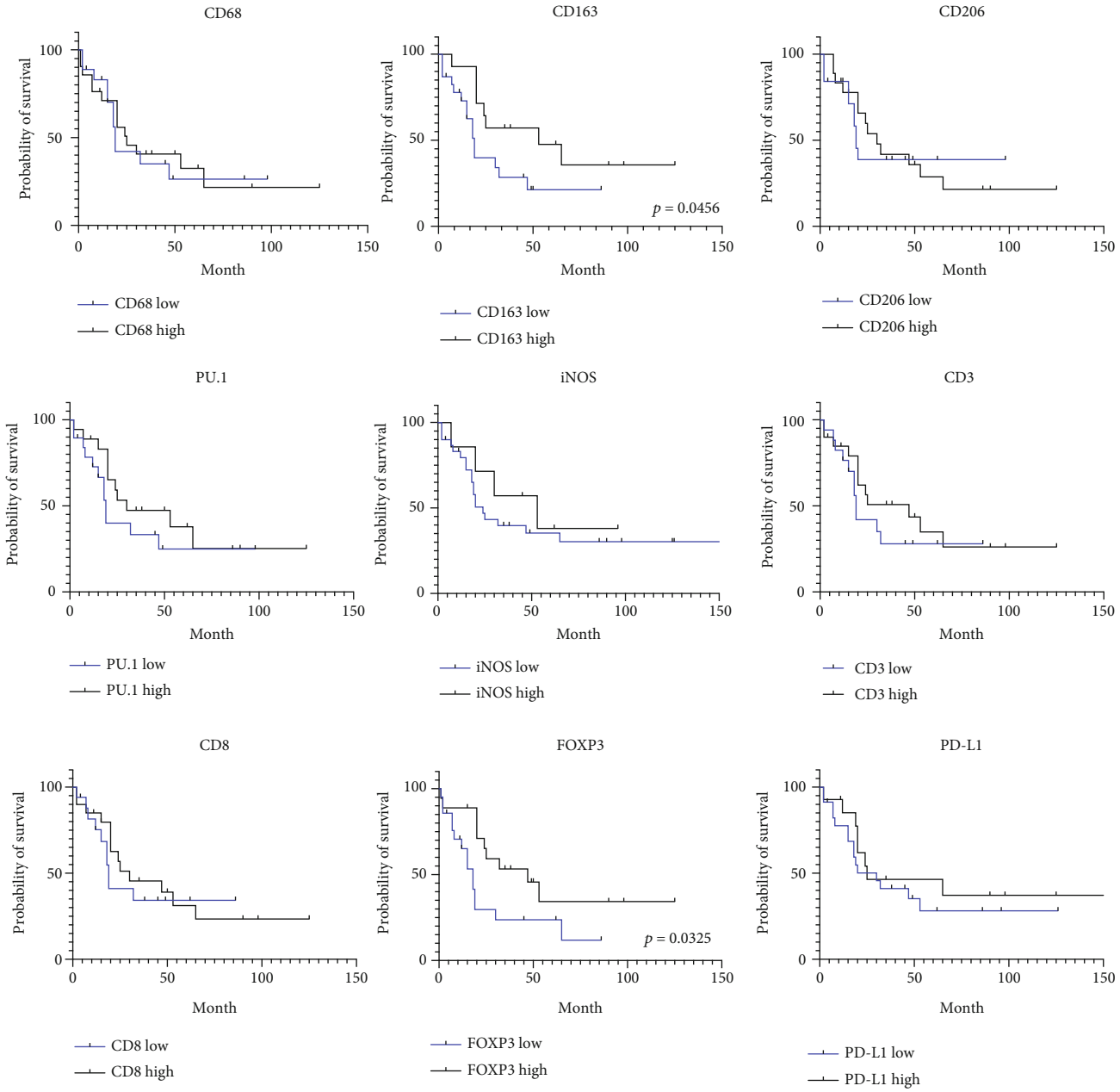

Figure 1: Kaplan–Meier curves of overall survival (OS) in esophageal squamous cell carcinoma (ESCC) based on TILs and TAMs.

also allow for multiplex analysis together with other macrophage markers.

We performed a correlation analysis of various macrophage markers in esophageal tumor and demonstrated that PU.1 expression strongly correlates with that of CD68 ($r = 0.833$, $p \leq 0.001$), CD163 ($r = 0.500$, $p \leq 0.001$), and CD206 ($r = 0.250$, $p = 0.043$) with the strongest correlation observed for CD68 (Figure 2).

IHC analysis of macrophage markers on serial tumor sections also demonstrates highly overlapping staining patterns for PU.1 and other macrophage markers (Figure 3). Strong correlation of PU.1 primarily with CD68 suggests possible usage of this marker as a general macrophage marker for tumor stroma.

4. Discussion

Tumor immune escape is an important aspect of tumor development that ensures tumor progression. Tumor cells produce soluble factors that modify microenvironment, attract various immune cells, and drive their differentiation to immunosuppressive phenotype. In this study, using various markers of tumor stroma cells, we investigated the immunosuppressive phenotype of esophageal squamous cell carcinoma (ESCC) (Figure 4).

The main cell population we have studied is composed of TAMs. Like other immune effector and regulatory cells, macrophages demonstrate high degree of functional versatility and express different surface markers and secretable factors

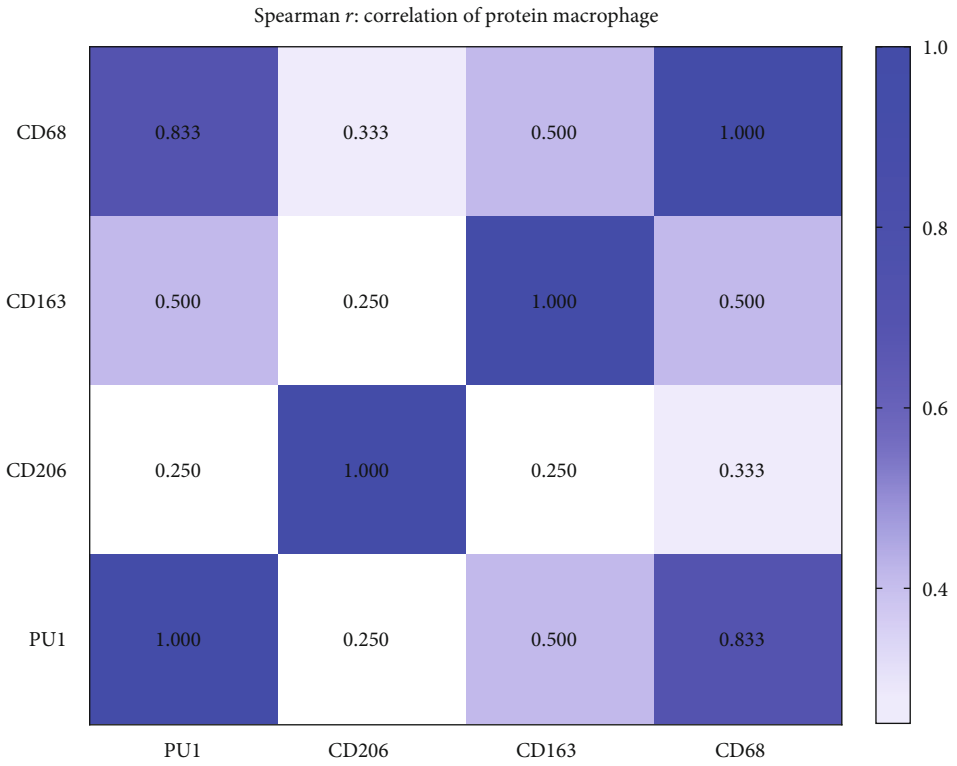

Figure 2: Spearman's rank correlation coefficient for macrophage markers.

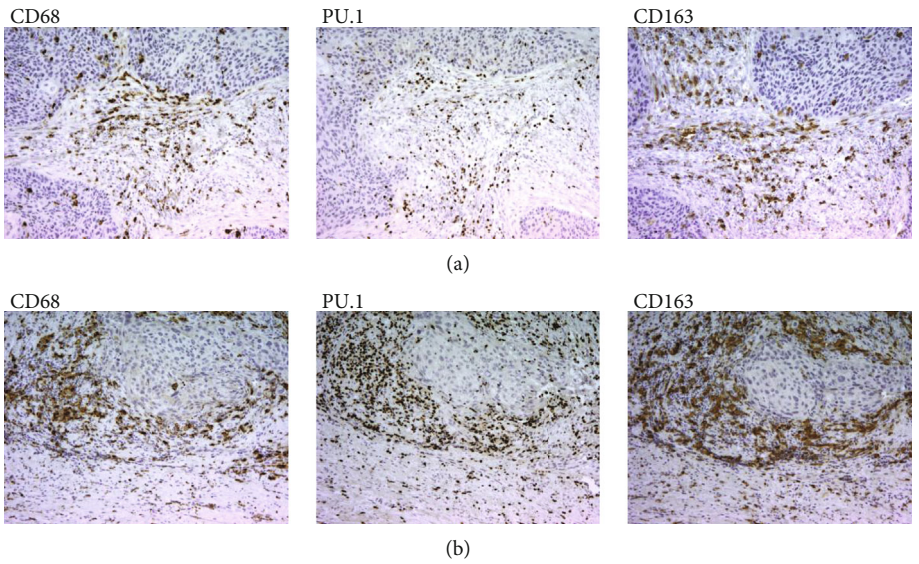

Figure 3: Immunohistochemical analysis of CD68, PU.1, and CD163 on serial tissue sections of 2 different tissue samples (magnification 100x).

[15]. The role they play in the tumor immune escape depends on their phenotype. Macrophages can be boldly divided into two main subgroups: "classically activated" or M1 and "alternatively activated" or M2. M1 are proinflammatory and are thought to exert antitumor effects through production of IL-12, IL-23, and reactive oxygen and nitrogen species [16]. M1 are not considered to be immunosuppressive; however, existence of mixed M1/M2 phenotype of TAMs prompted us to analyze M1 marker—iNOS. We found that this marker is not suitable for TAM analysis, since its expression was observed solely in tumor cells in a small number of samples. Interestingly, iNOS expression correlated with the stage of disease, and high expression was found in tumors of stages III–IV, compared to low expression at stages I–II. No prognostic value of iNOS was determined. iNOS expression in esophageal cancer is poorly studied. No significant correlation with the clinical parameters of the tumor and iNOS expression was found in the study by Jin et al. [17]; the

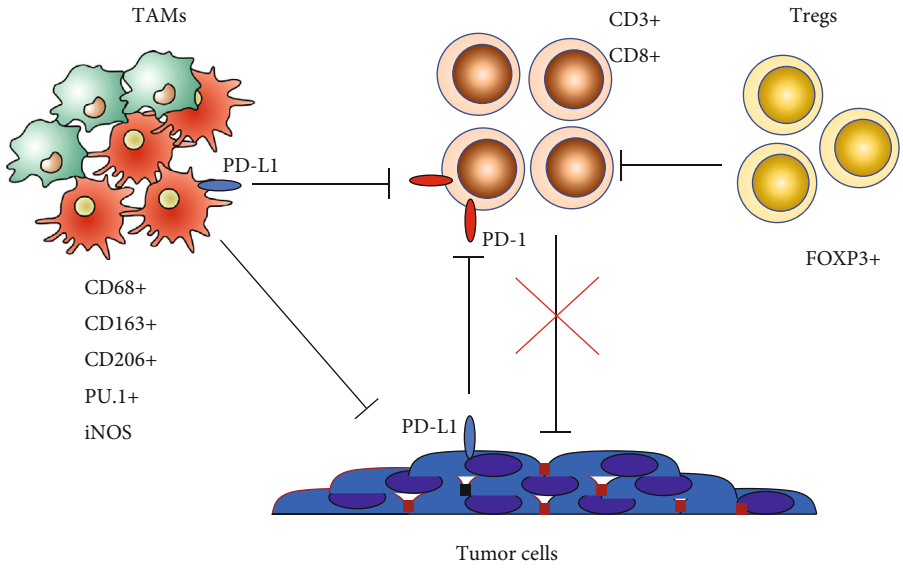

Figure 4: Immunosuppressive cells of esophageal squamous cell carcinoma stroma.

absence of expression difference between tumor and normal tissue of esophagus was also reported [18]. Our results, however, suggest that deeper investigation of iNOS in esophageal tumors will reveal its diagnostic and/or prognostic value.

M2 are usually considered to be able to suppress antitumor properties of M1 TAMs and modulate tissue remodeling by producing matrix metalloproteinases, transglutaminases, and extracellular matrix components [19] and various cytokines and growth factors [20]. In most of the tumors, TAMs have M2 phenotype that are considered to be tumor-supporting ones [21]. All tumor-associated macrophages independent on their phenotype seem to express CD68. A subtype-specific marker of M2 CD163 is frequently used [20]. However, there are several other markers like CD204, CD206, or Stabilin-1 that can be used to detect type 2 macrophages. We examined the relationship between TAM density and clinical characteristics and outcomes in 48 patients who had undergone resection of esophageal cancer. We demonstrated that out of all markers studied, only CD206 correlated with the histological grade of the tumor. No other correlations were found.

There are contradictory literature data regarding the prognostic value of M2 number in the tumor. In most of the cases, high number of M2 TAMs correlates with poor prognosis, since these macrophages promote vascularization, invasion, and metastasis in many cancer types [22]. In our study, the number of CD163+ M2 correlated with a good prognosis of esophageal cancer (HR = 0.4447, $p = 0.0456^*$). In contrast to our results, Hu et al. demonstrated correlation of stromal CD163+ TAMs with poor prognosis of esophageal cancer in a Kazakh population. In this study, however, the correlation was found only for the CD163+ cells counted in tumor stroma, while for CD163+ cells in tumor nests, no statistically significant correlation was found [13]. Another reason for observed discrepancy in results is a difference in study populations, with different genetic and cultural backgrounds. Similar contradictory data are available for some other malignancies. In the study by Edin et al. done on 485 samples of colorectal cancer, the higher numbers of CD163+ cells were clearly associated with a good prognosis [23]; a similar study on a cohort of 201 colorectal cancer patients that also demonstrated a tendency of better prognosis is the case of high CD163+ cell count, though these results were not statistically significant [24]. At the same time, there are studies demonstrating opposite correlations, i.e., poor prognosis of the colorectal tumors showing high amount of CD163+ cells [25]. Similarly, for gastric cancer, there are reports of high CD163 as an indicator of good and bad prognosis. Liu et al. have demonstrated that increased number of CD163+ cells is a marker of good prognosis of signet ring cell carcinoma and mucinous adenocarcinoma, while for other types of gastric cancer, it does not correlated with prognosis or is a marker of poor prognosis [26]. In the study by Cheng et al. where 139 gastric cancer cases were analyzed, a clear association of the high CD163 expression and poor prognosis was demonstrated [27]. Also, for different types of breast cancer, there is a difference in prognostic value of the number of CD163+ cells in tumor stroma. It was reported that high content of CD163+ cells is a marker of good prognosis in estrogen receptor negative breast cancer tumors [28]. These differences in published data clearly indicate the importance of the way how and in which areas of the tumor TAMs are analyzed. Furthermore, different prognostic values of CD163+ cells in different types of tumor of the same localization indicate that it can be strongly affected by specific tumor features that remain to be elucidated for esophageal cancer.

Since the total amount of macrophages is a highly important criterion, there is an urgent need for a macrophage marker that allows clear identification of the cell. We selected PU.1 as such a marker. PU.1 is a transcription factor regulating hematopoietic differentiation pathways [29]. Upon lineage differentiation and maturation, PU.1 is expressed at varied levels in mature blood cells, with higher levels found

in macrophages than B cells [30]. In our study, expression of PU.1 showed the strongest correlation with CD68 and a staining pattern indicating that the cells stained for PU.1 are CD68-positive macrophages. Taking into account nuclear staining, PU.1 will be more suitable for precise cell quantification.

In the present study, we also explored the impact of TILs on the clinical significance in ESCC. It was demonstrated that high numbers of TILs are a marker of good prognosis and longer survival in ESCC. Particularly, the presence of T cells (CD3+) and T cell subpopulations (e.g., CD4+, CD8+, and CD103+) was established to be markers of a good prognosis [31]. CD8+ T cells can recognize tumor-associated antigens as major histocompatibility complex (MHC) class I molecules on the cancer cell surface and lyse cancer cells. Therefore, the presence of CD8+ T cells in the tumor is considered a host immunoreaction and is associated with a better prognosis in a variety of cancers. However, opposite results are also reported, where high levels of CD8+ T cells in the tumor are associated with a poor prognosis [32]. In our study, we found no significant correlations of CD3+ and CD8+ cells and clinical features of the tumor. Also, analysis of prognostic value of T cells in general and cytotoxic T cells did not reveal statistically significant differences.

Another T cell type that has diagnostic and prognostic value in different types of cancer is regulatory T cells, expressing FOXP3. FOXP3 is a member of the forkhead/-winged-helix family of transcription factors that is critically involved in the development and function of Tregs [33]. Several studies demonstrate that FOXP3+ Tregs infiltrating tumor suppress CD8+ T cells to maintain immunological tolerance and associate with advanced tumor growth and poor prognosis in several types of malignant tumors [34–36]. In contrast, other studies have shown that tumor FOXP3 expression is a favorable prognostic factor for breast cancer [37, 38]. In the case of esophageal cancer, high numbers of FOXP3+ cells was reported to be an indicator of poor [39] as well as good [40] prognosis. In our study, we demonstrated that the high number of FOXP3+ cells is associated with good prognosis in the analysis of overall survival (HR = 0.4420, $p = 0.0325$).

5. Conclusions

In conclusion, data we obtained are in a good agreement with a number of studies, indicating that TAMs and TILs may provide important diagnostic and prognostic information for esophageal squamous cell carcinoma. However, discrepancies found suggest that there is a need for a general agreement on the methodology of stromal cell evaluation and specifically macrophage counting. Also, usage of a nuclear marker for macrophage identification can be recommended which will facilitate stromal cell identification.

Data Availability

All data that support conclusions made in the manuscript are included in the manuscript. Raw data, i.e., stained slides, are available for review in our laboratory.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

O. Kovaleva analyzed data and wrote the original draft preparation. P. Podlesnaya analyzed data. D. Samoilova, M. Rashidova, and V. Mochalnikova performed experiments. A. Gratchev was responsible for supervision, study conceptualization, writing, reviewing, and editing.

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Research Article

Expression of RAD51 and Its Clinical Impact in Oral Squamous Cell Carcinoma

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Purpose. To examine the expression of RAD51 in oral squamous cell carcinoma (OSCC) and analyze its connection with pathological grade, clinical stage, and lymphatic metastasis potential. *Methods*. For this study, 74 OSCC samples, 15 normal mucosa tissues, and 11 normal skin tissue samples were collected. RAD51 expression was investigated using immunohistochemistry. A follow-up visit was used to assess the prognosis of each patient. We compared RAD51 expression in oral mucosa epithelial cells (OMECs), keratinocytes, and tongue squamous cell carcinoma cells (TSCCs) by Western blot analysis. *Results*. RAD51 expression was higher in tumor cells than in normal mucosal tissues. In addition, RAD51 expression was associated with higher tumor differentiation ($P < 0.05$). Also, RAD51 expression was higher ($P < 0.05$) in patients with lymphatic metastases, and relapse rates were also higher in patients with elevated RAD51 levels ($P = 0.052$). In addition, RAD51 expression levels were highest in the skin keratinocytes, followed by the TSCCs and OMECs. *Conclusion*. A strong positive correlation was found between RAD51 expression and the degree of malignancy in OSCC patients, suggesting that RAD51 could be an excellent prognostic indicator for OSCC patients.

1. Introduction

Oral squamous cell carcinoma (OSCC) accounts to more than 90% of all oral malignancies diagnosed each year. Globally, it is the most common form of cancer that arises from the mucosal membranes of the oropharynx and mouth with more than 275,000 new cases diagnosed each year and 125,000 deaths attributed to this malignancy each year worldwide [1]. Due to the rich lymphatic network in the maxillofacial region, OSCC displays a high metastatic potential with more than 40% of OSCC patients developing cervical lymph node metastases within two years of their initial diagnosis [2]. Once the malignancy spreads to the surrounding lymph nodes, patient survival rates decline significantly with only 40-50% of individuals surviving five-years after the initial diagnosis. However, the survival rate for patients without metastases is 90%, suggesting that the key to improving survival rates is the early diagnosis and prompt treatment of the disease. With this in mind, scientists have identified several biomarkers that could be useful for identifying early-stage disease in symptomatic patients or high-risk individuals [3].

RAD51 is a 339-amino acid that plays an essential role in repairing DNA double-strand breaks. When the double-stranded structure of DNA is injured, RAD51 exploits a sister DNA molecule as a template that allows for homologous recombination (HR) in the damaged region, which maintains the stability of the genes [4]. While RAD51 displays a protective role, scholars have found that RAD51 is overexpressed in several tumor types, including breast, pancreatic, head and neck, prostate, non-small cell lung, and esophageal cancers [5, 6]. Recently, Yuan et al. [7] discovered that high RAD51

expression was directly associated with increased chemo- and radio-resistance, along with dismal outcomes and prognoses in breast cancer patients. In another study, Chen et al. [8] revealed that RAD51, which was overexpressed in patients with cervical cancer, promoted the differentiation of cancer cells from the G0/G1 to S phase. Interestingly, inhibition of RAD51 increased the sensitivity of the cancer cells to radiotherapy, which is a potential therapeutic strategy that requires further exploration. Furthermore, the results from a recent meta-analysis revealed that high RAD51 expression could increase the risk of patients developing head and neck tumors [9].

The current literature shows that radiotherapy after surgery is an ideal treatment for squamous cell carcinoma of the head and neck for improving the overall survival and quality of life for patients. However, radiotherapy causes off-site injury to healthy tissues, which can lead to unwarranted inflammation or the development of mucosal ulcers [10]. The radiation will also negatively impact the epidermal cells and oral mucosa epithelial cells, yet the adverse effects associated with these cells are less severe than the mucosa [11]. Therefore, we believe that RAD51 may play an important role in resisting radiation-induced damage.

Currently, limited studies have assessed the role of RAD51 in OSCC. In this study, we aimed to evaluate the potential use of RAD51 as a prognostic indicator of OSCC. To do this, we examined RAD51 expression in OSCC, oral mucosa, and skin tissue samples and analyzed its association with lymphatic metastasis. We also compared RAD51 expression in oral mucosa epithelial cells (OMECs), keratinocytes, and tongue squamous cell carcinoma cells (TSCCs) to provide a theoretical basis for the clinical use of RAD51 as a prognostic biomarker for oral cancer.

2. Material and Methods

2.1. Patient Samples. The study was approved by the Ethics Committee of Jilin University. Prior to the study, all patients provided written informed consent. For this study, the Department of Oral Pathology provided 74 OSCC tissue samples, 15 adjacent normal tissue samples, and 11 normal skin tissue samples from patients treated at the Hospital of Stomatology, Jilin University (Changchun, China), between 2013 and 2017. Those patients with a history of diabetes, hypertension, or systemic, metabolic, and immunological diseases were excluded from the study. In addition, the consumption of alcohol and cigarette smoking were also exclusion criteria. None of the patients received prior treatments, including surgery, radiotherapy, or chemotherapy before the surgery at the Hospital of Stomatology, Jilin University. Of the patients, 55 were male (74.3%) and 19 were female (25.7%) with a mean age of 57.2 years (range: 33-81). Histologically, 21 cases were well-differentiated, 32 were moderately differentiated, and 21 were poorly differentiated cases of OSCC. A total of 28 cases (14 + 14, 37.8%) had early-stage disease (I + II), while 46 cases (22 + 24, 62.2%) had advanced-stage disease (III + IV). Furthermore, 21 cases (28.4%) had lymphatic metastases whereas 53 cases (71.6%) had no lymphatic metastases (Table 1). Patient samples were collected after surgery and sectioned at 4 μM thickness after being fixed in 10% formalin, dehydrated, and paraffin embedded. The sections were adhered to slides and used for histological studies.

Table 1: Gender and clinical data about OSCC patients.

Variables	# of patients	% of patients
Gender		
Male	55	74.3
Female	19	25.7
Histological grade		
Well	21	28.4
Moderate	32	43.2
Poor	21	28.4
Clinical stage		
I	14	18.9
II	14	18.9
III	22	29.7
IV	24	32.5
Lymphatic metastasis		
No	53	71.6
Yes	21	28.4

2.2. Immunohistochemistry. The OSCC and normal tissue samples were subjected to immunohistochemistry using a commercial kit (MXB Biotechnologies, Fuzhou, China). The optimal concentration of polyclonal antibodies against RAD51 (ABclonal Biotech Co., Ltd, Wuhan, China) was 1 : 100. According to the protocol outlined by the manufacturer, the sections were deparaffined in xylene, graded alcohol, and tap water. Next, the sections underwent microwave antigen retrieval in a citric acid solution (pH 6.0) for 10 min before incubating in the endogenous peroxidase blocker (3% H_2O_2) for 10 min. After washing with PBS, the sections were incubated with normal sheep serum at room temperature for 20 min. Then, the primary antibody diluted in PBS was added for an overnight incubation at 4°C. The sections were washed in PBS and incubated with sheep anti-rabbit IgG for 10 min. After washing in PBS, the sections were incubated in biotin-labeled streptavidin (10 min) and subjected to a color reaction using DAB kit (MXB Biotechnologies, Fuzhou, China) for 3 min and counterstaining in hematoxylin for 1 min. Next, the sections were processed by hydrochloric acid alcohol, and ammonia. Finally, the sections were dehydrated in graded ethanol solutions, cleared in xylene, and mounted with neutral balsam using coverslips.

2.3. Immunohistochemical Scoring. The stained sections were viewed and scored under a light microscope by two independent investigators. Each section was evaluated at five fields at 400x magnification. The presence of a clearly visible yellow or brown precipitation was considered an immunoreaction, and each section was scored according to the degree of positive staining and the staining intensity. The samples were classified as negative (0 as 0-10% positivity) or positive

(1 as 10-25% positivity, 2 as 25-50% positivity, 3 as 50-75% positivity, and 4 as 75-100% positivity). Expression intensity was evaluated semiquantitatively without prior knowledge of any clinical information using a four-level system (0 as negative, 1 as weak, 2 as moderate, and 3 as strong) [12]. The staining index for each section was then reached by multiplying the positive cell score by the intensity score to obtain a final score, which was an average of five scores. For statistical analysis, the samples were categorized into two groups: negative (score ≤ 5) and positive (score > 5) [13].

2.4. Cell Culture. The TSCC cell lines, CAL-27 and SCC-9, were purchased from ATCC (Manassas, VA, USA). Human keratinocytes (HaCaT) were provided by Professor Hongchen Sun (Department of Oral Pathology, Hospital of Stomatology, Jilin University). OMECs were isolated from the excessive mucosal tissues of a healthy donor (male, 23 years) in an orthognathic surgery from the Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Jilin University. The donor provided written informed consent prior to this study. The mucosal tissues were washed with PBS, and the epithelial part was isolated and cut into small fragments (1 mm^3) before digesting in 2.5 g/L Dispase II (Solarbio Science & Technology Co., Ltd., Beijing, China). After washing in PBS, the epithelium was digested in the mixed liquor containing 0.25% trypsin (Invitrogen) and 0.03% EDTA (Invitrogen) and blocked in DMEM to prepare monoplast suspension.

CAL-27 cells were maintained in RPMI-1640 (Invitrogen) and supplied with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 100 U/mL penicillin, and 100 mg/L streptomycin. SCC-9 cells were incubated in DMEM/F-12 (Invitrogen) with the supplements of 10% FBS and 400 μg/L hydrocortisone (Sigma). HaCaT and oral mucosa epithelial cells were cultured in Keratinocyte Serum Free Medium (K-SFM; Gibco). All of the cells were maintained at 37°C in a humidified incubator with 5% CO_2. The experiments were carried out using cell growth in the logarithmic phase.

2.5. Western Blot Analysis. Cells were washed with PBS and lysed in RIPA buffer (Millipore, Billerica, MA). Protein concentration was determined with the bicinchoninic acid protein assay. First, 40 μl of total protein was resolved using a 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk powder for 1.5 h, the membranes were incubated with primary antibodies for RAD51 (Cell Signaling Technology, Shanghai, China) at 4°C for overnight. After washing, the membranes were treated with the appropriate horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence systems (GE Healthcare, London, UK). β-Actin was used for normalization.

2.6. Clinical Follow-Up Visits. Follow-up visits were carried out with select patients for more than five years after surgery. During the follow-up visits, patient characteristics, treatment plans, and cases of relapse were recorded.

Table 2: RAD51 expression in OSCC patients.

Variables	Positive number (%)	*P* value
Gender		
Male	26 (47.3)	0.275
Female	7 (36.8)	
Property		
Oral mucosa	3 (20)	< 0.05
OSCC	33 (44.6)	
Skin	7 (63.6)	
Histological grade		
Well	5 (23.8)	< 0.05
Moderate	14 (43.8)	
Poor	14 (66.7)	
Clinical stage		
I + II	9 (32.1)	0.055
III + IV	24 (52.2)	
Lymphatic metastasis		
Yes	13 (59.1)	0.027
No	20 (38.5)	

2.7. Statistical Analysis. All statistical analyses were performed using SPSS version 18.0 (IBM, Chicago, IL, USA). Comparisons between groups were assessed using the one-way analysis of variance (ANOVA), Student's t test, or χ^2 test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. RAD51 Expression between the OSCC and Healthy Tissue Samples. RAD51 expression was mainly localized on the cytoplasm and a few on the nucleus of cells. The between-group comparisons are shown in Table 2. RAD51 expression was significantly stronger in OSSC tissues when compared with normal tissues, and RAD51 expression levels were positively correlated with the disease stage ($P < 0.05$; Figure 1), suggesting that RAD51 expression could be potentially used to estimate the prognoses of OSCC patients. In addition, RAD51 expression was also higher in OSCC patients with lymphatic metastases ($P = 0.027$). Lastly, according to the TNM stage, we found that RAD51 expression was higher in patients with advanced-stage disease (III or IV) when compared with those patients with early-stage (I or II) OSCC ($P = 0.055$), yet this finding was not statistically significant.

3.2. RAD51 Expression Levels in TSCC Cells Were Higher than Those in Oral Mucosa Epithelial Cells and Lower than those in HaCaT Cells. Western blot analysis was used to assess the RAD51 expression levels among the TSCC cells, OMECs, and skin cells. RAD51 was differentially expressed in all of the cell lines. As shown in Figure 2, RAD51 expression levels were highest in the in the skin keratinocytes (HaCaT), followed by the TSCC cells (CAL-27 and SCC-9). The OMECs showed the lowest levels of RAD51 expression.

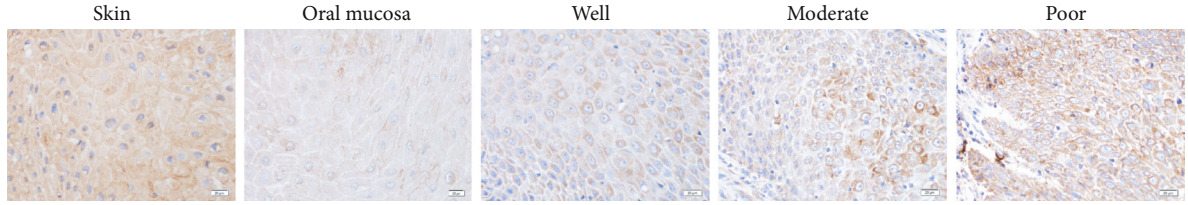

Figure 1: Expression of RAD51 in OSCC tissues (×400). RAD51 expression was lower in normal tissues than in OSCC tissues ($P < 0.05$). RAD51 was highest in the poorly differentiated OSCC tissues and lowest in the well-differentiated OSCC when compared with the healthy control tissues ($P < 0.05$).

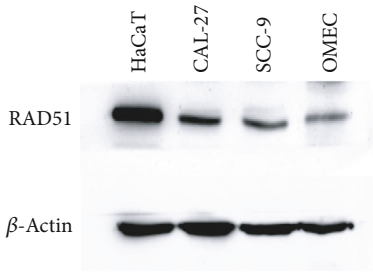

Figure 2: Expressions of RAD51 in different cell lines. Western blot analysis showed the RAD51 expression was highest in the HaCaT cells, followed by TSCC cells (CAL-27 and SCC-9), and oral mucosal epithelial cells.

Table 3: RAD51 expression is linked to dismal patient survival and patient relapse.

Variables	Number of patients (%)	*P* value
Death	18 (47.4)	0.036
Relapse	19 (50)	0.052
Relapse with radiotherapy	13 (43.3)	0.047
Relapse without radiotherapy	6 (75)	
Well-differentiated	3 (15.8)	<0.01
Moderately differentiated	12 (63.1)	0.073
Poorly differentiated	4 (21.1)	0.024

The reparability of TSCC cells was stronger compared to OMECs.

3.3. Follow-Up Visits. There were total 39 patients who met the time standards, of which one patient lost touch and 38 patients provided the relevant information (Table 3). Of the 38 patients, eighteen were deceased by the follow-up and 19 patients had relapsed. RAD51 expression was significantly higher in the samples from patients who were deceased by the follow-up visit ($P = 0.036$). Also, there was a high tendency of RAD51 expression in patients who have recurrent disease when compared with patients with nonrecurrence ($P = 0.052$), yet this finding was not statistically significant. In order to further explore the function of radiotherapy in postoperative patients, we analyzed the cases of disease recurrence. Results showed that the relapse rate in the radiotherapy group (43.3%) was lower than that in patients who did not receive radiotherapy (75%, $P = 0.047$). In recurrent cases, we also compared RAD51 expression among the well, moderately, and poorly differentiated groups. In all the groups, including the well ($P < 0.01$), moderately ($P = 0.073$), and poorly differentiated ($P = 0.024$), RAD51 expression was higher in those patients with the relapsed disease when compared with those patients without relapsed disease. Overall, high expression of RAD51 indicated a poor prognosis and a high possibility of disease recurrence in OSCC patients.

4. Discussion

In recent years, RAD51 expression has been detected in several types of cancer, including mainly breast cancer [14, 15], cervical cancer [15, 16], and ovarian cancer [17]. The homozygous gene RAD51 GG was found to be most often in breast cancer patients [16], while RAD51 G172T has the most clinical significance in cervical cancer [18, 19]. In this study, we investigated the possible link between RAD51 expression and OSCC and found that RAD51 was expressed higher in OSCC patients. In addition, we discovered that RAD51 expression was significantly higher in poorly differentiated tissues compared with moderately or well-differentiated tissues (Figure 1, Table 3) and that patients with advanced-stage disease showed stronger levels of RAD51 when compared with patients with early-stage OSCC ($P = 0.055$). These findings suggest that RAD51 may be a viable biomarker for staging OSCC in the future [20].

Overexpression of RAD51 can result in the formation of genotoxic RAD51 protein complexes on undamaged chromatin, which will decrease the efficiency of homologous recombination [21]. Also, RAD51 overexpression can lead to the enhancement of cell growth inhibition and apoptotic induction, resulting in tumor progression [22]. Furthermore, RAD51 can protect tumor cells from radiation-induced damage. Previously, An et al. [23] used T0070907 (T007), one kind of peroxisome proliferator-activated receptor gamma (PPARγ), to weaken the expression of RAD51 in cervical cancer and disturb the mitosis of tumor cells. They found that the agent also increased the sensitivity to radiation therapy. In another study, Liu et al. [24] knocked-down the RAD51 gene in triple-negative breast cancer (TNBC) cells, which were found to inhibit cell proliferation. In addition, the cancer stem cells (CSCs) in the TNBC could resist poly ADP-ribose polymerase (PARP) inhibitors. However, when

the RAD51 gene was knocked-down in the CSC, its sensitivity to PARP inhibitors and radiation therapy increased, which effectively inhibited tumor growth [25].

In the current study, we analyzed a potential connection between RAD51 expression and OSCC. Our findings demonstrated that high expression of RAD51 was indicative of poor patient prognoses (Table 3). In immunohistochemistry studies, RAD51 expression in the well-differentiated group was lower than the moderately and poorly differentiated groups. Compared to those patients who did not have a recurrence of OSCC by the time of the follow-up visit, whatever in the well ($P < 0.01$), moderately ($P = 0.073$), or poorly ($P = 0.024$) differentiated group, higher levels of RAD51 could be shown in the recurrent case samples. All of the above findings indicated that increased expression of RAD51 was closely related to the occurrence, development, and relapse of OSCC.

Furthermore, the upregulation of DNA repair protein, like RAD51, could reduce the radiation-induced DNA damage [26]. Considering the derivation of OSCC, we compared the expression levels of RAD51 among TSCCs, OMECs, and keratinocytes and found that RAD51expression was highest in the skin, followed by the OSCC, and oral mucosa (Figure 1, Table 2). Cell-based experiment showed similar findings (Figure 2), indicating that skin tissue has a stronger ability to resist radiation-induced damage. This explains why oral mucosa is ulcerogenic during the radiotherapy [10], while the effects on the skin are less pronounced. Nonetheless, skin tissues need more time to recover from the radiation-induced damage than oral mucosal tissues [27], yet the exact mechanisms remain to be elucidated.

In addition, high RAD51 expression was found to be correlated with increased metastatic potential. Recently, Mahdi et al. [28] examined RAD51 expressions in primary ovarian tumors and metastatic and discovered significantly higher expression in the metastatic tumors. High expression of RAD51 in breast cancer was previously shown to increase the risk of brain metastases and micrometastases. However, the knockdown of RAD51 decreased the metastatic potential of breast cancer [29]. Scholars have confirmed that high RAD51 expression could function as a critical factor in the promotion of lymphatic metastases from tumors [30, 31]. As a confirmation of that finding, this study showed that RAD51 expression was significantly higher in OSCC patients with lymphatic metastases ($P < 0.05$).

This study is the first to report on the high expression of RAD51 in OSCC patients, which was compared with the oral mucosa and skin. While the RAD51 protein has a recovery effect, it can also enhance the antidamage and invasion abilities of tumor cells because of its nonselective properties. Therefore, RAD51 may be viewed as an important protein for future targeted therapies. Overall, we found that RAD51 was an excellent prognostic indicator for patients with OSCC.

Data Availability

The data and materials are available on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

WL and YYL conceived and designed this study. YYL, JL, and JS performed the experiments. KYL, DL, LD, and BW collected and analyzed the data. YYL and JL prepared the manuscript. WL, YYL, and JS revised and drafted this manuscript. All authors read and approved the final manuscript. WL provided the funding support in the study. YYL and JL contributed equally in this work.

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