Overexpression of HIF-1α and high density of tumor associated macrophages predict tumor aggression, invasion and unfavorable prognosis of Kazakh esophageal squamous cell carcinomas

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Abstract

Background

A hypoxic tumor microenvironment promotes the recruitment and transformation of macrophages and facilitates tumor progression. Hypoxia-induced factor-1α (HIF-1α) is a key factor in hypoxia. It is not clear whether HIF-1α and tumor associated macrophages (TAMs) play a synergistic role in neoplastic progression. Here we investigated potential synergism between HIF-1α and TAMs in the progression of Kazakh esophageal squamous cell carcinoma (ESCC).

Methods

We used immunohistochemistry (IHC) verify the expression of HIF-1α and vascular endothelial growth factor (VEGF) in tumor tissue. CD163 was used as a marker for TAMs, and the density of TAMs in tumor tissues were determined. Kazakh ESCC samples exhibited higher expression of HIF-1α and VEGF and greater density of TAMs compared to cancer adjacent normal (CAN) tissues.

Results

Overexpression of HIF-1α was significantly correlated with vascular invasion, lymph node metastasis and the clinical tumor stage of Kazakh ESCCs. Overexpression of HIF-1α was also associated with poor prognosis of Kazakh ESCC. Moreover, overexpression of HIF-1α was positively correlated with high TAM density and overexpression of VEGF ($P < 0.05$). Furthermore, the correlation between VEGF overexpression and high density of TAMs was very significant ($P < 0.001$).

Conclusions

These data suggest that overexpression of HIF-1α may increase the infiltration of TAMs in Kazakh ESCC, thereby increasing VEGF expression, which may in turn promote the progression of Kazakh ESCC.

Background

Esophageal carcinoma is one of the most common malignant tumors in the world. The incidence and mortality rate of esophageal cancer in China are high; in particular, the Kazakh national ethnic minority in Xinjiang (northwest of China) exhibits the highest incidence of ESCC, which is higher than the average rate in China of 15.23/100,000 [1]. According to its etiological and pathological features, esophageal carcinoma is divided into two major types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). In China, ESCC comprises more than ninety percent of all esophageal carcinomas. Despite great improvements in diagnosis and treatment, the prognosis for ESCC patients remains poor due to high rates of recurrence, metastasis, and resistance to adjuvant therapy [2,3].

Hypoxia is one of the most common stresses in the tumor microenvironment [4], and hypoxia-inducible factor-1 (HIF-1) plays a crucial role in response to hypoxic stress [5]. The HIF-1 transcription factor complex consists of two subunits; an oxygen-sensitive HIF-1α subunit and a constitutively active HIF-1β subunit [6]. HIF-1α is overexpressed in many cancers, including gastric cancer, breast cancer, prostate cancer, and colon cancer. Furthermore, overexpression of HIF-1α significantly influences the tumor cell biology, and modulates the recruitment of immune and inflammatory cells in tumor microenvironment [7,8]. Macrophages are the most abundant immune cell type recruited to the tumor microenvironment of hypoxic tumors.

Macrophages can be phenotypically polarized by the microenvironment to exhibit characteristics of classically activated macrophages (M1 macrophages) or alternatively activated macrophages (M2 macrophages) [9]. M1 macrophages exhibit potent microbicidal properties, while M2 play a detrimental and pro-tumor role, and are also called tumor-associated

macrophages (TAMs) [10,11]. Our previous studies demonstrated that TAMs in Kazakh ESCCs are closely associated with tumor invasion and metastasis [12,13]. The hypoxic microenvironment exhibits stably elevate expression of HIF-1α, and M2 TAMs comprise the primary immune cell components of the hypoxic tumor microenvironment. However, whether HIF-1α and macrophages play a synergistic role in the progression, invasion, metastasis of Kazakh ESCC is not clear. In the present study, we examined the distribution of HIF-1α and M2 TAMs in Kazakh ESCC tissues. We evaluated the association of HIF-1α expression and M2 TAM distribution with patient clinical parameters, pathological parameters, and outcome data to explore the potential synergistic effects of HIF-1α and M2 TAMs in the invasion, metastasis, and prognosis of Kazakh ESCC.

Methods

Patients and specimens

Two hundred cases of therapeutic resection and paraffin-embedded human tissue were obtained from the Department of Pathology of Xinjiang Yili Friendship Hospital, including 100 cases of Kazakhstan ESCC and 100 cases of Kazakh adjacent normal tissue (CANs) (samples were collected from 2008 to 2014). Each participant provided written informed consent, and the study was approved by the participating hospital. Of the cancer patients, sixty-four were male and thirty-six were female, their ages ranged from thirty-four to seventy-four years old, all had been diagnosed with ESCC, but none had received radiotherapy or chemotherapy prior to surgery. CAN group participants consisted of fifty-six males and forty-four females, ranging in age from thirty-three to seventy-three years old. All tissue specimens were cut into 5 μm sections and subjected to conventional hematoxylin and eosin staining. Tumor pathological diagnosis was performed by two independent pathologists and according to the World Health Organization's histological tumor classification. Among the ESCC cases, thirty-two cases were well-differentiated, forty-eight cases were moderately differentiated, and twenty cases were poorly differentiated. Of the ESCC cases, thirty-three cases had an invasion depth of T1–T2 and sixty-seven cases had an invasion depth of T3–T4. There were forty-eight cases with lymph node metastasis, fifty-two without lymph node metastasis. Sixty-three cases were clinical stage I or II, and thirty-seven cases were clinical stage III or IV. CAN specimens were sampled more than 5 cm away from the cancer region, and were confirmed to have no cancerous tissue. The health status of all patients was followed until December 2015. The median follow-up time for surviving patients was 26 months (range 1–78 months). The overall survival (OS) was defined as the interval between surgery and death or between surgery and the final follow-up for living patients. Of the 100 patients, 55 (55.0%) died of tumor-related causes and 45 (45.0%) were alive at the time of final follow-up.

Immunohistochemistry

Paraffin-embedded tissues sections were cut into 4 μm thick slices and mounted on polylysine-coated slides. The samples were dewaxed in xylene and rehydrated through a series of graded ethanol solutions. After dewaxing, endogenous peroxidase activity was inhibited by incubation in a 3% peroxide-methanol solution for 10 minutes at room temperature (RT). Antigen retrieval was carried out in an autoclave at 100° C for seven minutes. The samples were then incubated for 30 minutes at room temperature. The sections were then washed three times in phosphate buffered saline (PBS) for five minutes each time. Samples were then incubated with rabbit anti-human HIF-1α monoclonal antibody (1:200 dilution; Abcam, USA), mouse anti-human VEGF monoclonal antibody (1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-human CD163 antigen monoclonal antibody (1:100 dilution; Zhongshan Goldenbridge Biotechnology Co., LTD., Beijing, China). Samples were then thoroughly washed with PBS, and primary antibody binding was visualized using a DAKO EnVision kit (DAKO, Glostrup, Denmark), according to the manufacturer's instructions. Finally, sections were faintly counter-stained with hematoxylin and mounted with glycerol gelatin. For negative staining controls, PBS was used instead of primary antibody.

Immunoreactivity evaluation

HIF-1α and VEGF immunohistochemistry (IHC) reactivity were evaluated referring to previously described methods [7]. Positive IHC stains were defined as yellow-brown color, in accordance with the manufacturer's guidelines (Fig. 3). Tissue sample stained for IHC were scored as positive or negative based on the percentage and intensity of positively stained cells; the percentage of positively stained cells was classified as 0, 1, 2, 3, and 4, representing positive staining in ≤ 5%, 6–25%, 26–

50%, 51–75%, and 76–100% of cells; staining intensity was classified as 0, 1, 2, and 3, corresponding to absent, weak, moderate, and strong staining. A final score was determined by multiplying both scores from an individual slide (Table 1), where: 0–1 was negative (−), 2–3 was weakly positive (1+), 4–6 was moderately positive (2+), and 8–12 was strongly positive (3+). We divided these four classifications of expression into two groups: a low expression group (−/1+) and a high expression group (2+/3+).

Table 1
Scoring of HIF-1 and VEGF IHC

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Staining % positive cells		Staining intensity		Final score product	
Percent (%)	Score1	Intensity	Score2	Score1 × Score2	Score3
< 5%	0	Absent	0	0–1	0(-)
6%-25%	1	Weak	1	2–3	1(1+)
26%-50%	2	Moderate	2	4–6	2(2+)
51%-75%	3	Strong	3	8–12	3(3+)
76%-100%	4				

Note: Slides stained with IHC were scored as positive or negative by the percentage and intensity of positive cells. Scoring the percent of positively stained cells: Score 1 (0 = < 5%; 1 = 6%-25%; 3 = 51%-75%; and 4 = 76%-100%). Scoring the intensity of the staining: Score 2 (0 = absent; 1 = weak; 2 = moderate; 3 = strong). For final scores: 0–1 was negative (-), 2–3 was weakly positive (1+), 4–6 was moderately positive (2+), and 8–12 was strongly positive (3+).

The numbers of CD163-positive macrophages were analyzed as described previously [14]. For evaluation of IHC staining, the five most representative hot spots from low-power fields (LPFs,100×) per slide were selected used an Olympus BX51TF microscope (Olympus, Japan). The tumor nest and stroma areas were defined, and the numbers of CD163-positive macrophages were counted in high-power fields (HPFs, 400×) by two independent pathologists blinded to patients clinical data. If cell counts differed by over 10 cells per HPF, samples would be recounted after one week, until the independent counts were within 10 counts. The average number of macrophages per HPF across five hot spots for each sample (tumor nest and tumor stroma) was defined as the TAMs density.

Bioinformatics analyses

The ONCOMINE database (www.oncomine.org) was used to evaluate differential gene expression of HIF-1α and CD163 between cancer samples and corresponding normal control esophagus tissue. Differences with $P < 0.05$ were considered to be statistically significant. Heat maps of HIF-1α, CD163, and VEGF expression were generated using R heatmap package.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. Nonparametric test was used to compare the difference of HIF-1α, VEGF and CD163 expression between ESCC and CAN groups. The Chi square test or the Fisher's exact test were to analyze the correlation between HIF-1α expression and clinicopathological parameters. The cumulative survival rate was calculated by the Kaplan–Meier method and Cox proportional hazards modeling. Univariate and multivariate analyses were performed using the Cox proportional hazard regression model. Spearman's rank correlation method was used to evaluate the correlations between the HIF-1α and both CD163-positive TAMs and VEGF. Each cell experiment was repeated at least 3 times, and the differences between the two groups were tested by Student's test. p−Values were calculated using the Epi-Info program, and P values less than 0.05 were considered significant in all analyses.

Results

Evaluating HIF-1α expression in Kazakh ESCCs and CANs, and exploring its association with clinicopathological parameters and prognosis in Kazakh ESCC

IHC staining indicated that HIF-1α was generally present in the cytoplasm of cancer cells, and the percentage and intensity of positively stained cells varied greatly among cases (Fig. 1A-D). HIF-1α positive cells were diffusely distributed in tumor nests, and tumor stromal cells exhibited little HIF-1α expression. Only 9.0% of ESCC tissue cases were negative for HIF-1α antibody staining, and 50.0% cases showed strong staining (2+/3+). In contrast, 40.0% of CAN cases were negative for HIF-1α staining, and only 35.0% of CAN tissues showed strong staining (2+/3+) ($\chi^2 = 29.659$, $P < 0.001$, Table 2).

Table 2
The Expression of HIF-1 in Kazakh ESCC and CAN tissues

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Tissue Type		Negative			Positive				
	N	0(%)	1+ (%)	N1	2+ (%)	3+ (%)	N2	χ^2	*P*
ESCCs	100	9	41	50	34	16	50		
		9.0%	41.0%		34.0%	16.0%		17.120	< 0.000*
CANs	100	40	39	79	19	2	21		
		40.0%	39.0%		33.0%	2.0%			
Note: ESCCs: Esophageal squamous cell carcinoma tissues. CANs: Cancer adjacent normal tissues. $P < 0.05$.									

Using the Oncomine esophagus sample data, we evaluated the correlation between HIF-1α expression and ESCC occurrence, and we found that the expression of HIF-1α was significantly higher in ESCC than in normal tissue (Fig. 1E). In our study, we also found that expression of HIF-1α was higher in Kazakh ESCC than in CAN tissue (Fig. 1F). In order to better compare differential expression of HIF-1α to clinical parameters of ESCC, we divided the cases into two groups based on HIF-1α expression; a low HIF-1α expression and a high HIF-1α expression group. We found that cases with overexpression of HIF-1α showed more vascular invasion (60.0% vs. 26.7%, $P = 0.005$), lyphoid metastasis (pN^+ vs pN^- = 72.9% vs. 28.8%, $P < 0.001$, Table 3), and present at more advanced ESCC stages (III–IV vs. I–II = 81.1% vs. 31.7%, $P < 0.001$). These results indicated that HIF-1α expression may be associated with invasion and progression of Kazakh ESCC.

Table 3
Correlation between expression of HIF-1 and clinicopathological parameters in Kazakh ESCC

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Variable	Cases	HIF-1α low expression	HIF-1α high expression	χ^2	*P*	VEGF low expression	VEGF high expression	χ^2	*P*
	N	0/1+ (%)	2+/3+ (%)			0/1+ (%)	2+/3+ (%)		
Age(y)									
≤Median(58y)	53	26(49.1%)	27(50.9%)	0.000	1.000	15(28.3%)	38(71.7%)	0.108	0.742
Median	47	24(51.1%)	23(48.9%)			11(23.4%)	36(76.6%)		
Sex									
Male	64	29(45.3%)	35(54.7%)	1.085	0.298	9(14.1%)	55(85.9%)	11.50	0.001*
Female	36	21(58.3%)	15(41.74%)			17(47.2%)	19(52.8%)		
Tumor location									
Upper	1	1(100%)	0(0%)	1.094	0.579	0(0%)	1(100.0%)	0.407	0.816
Middle	74	36(48.6%)	38(52.4%)			19(25.7%)	55(74.3%)		
Low	25	13(52.0%)	12(48.0%)			7(28.0%)	18(72.0%)		
Histologic grade									
Well	32	16(50.0%)	16(50.0%)	4.631	0.099	6(18.8%)	26(81.3%)	1.343	0.511
Moderate	48	28(58.3%)	20(41.7%)			14(29.2%)	34(70.8%)		
Poor	20	6(30.0%)	14(70.0%)			6(30.0%)	14(70.0%)		
Depth of invasion									
T1-T2	33	20(60.6%)	13(39.4%)	1.628	0.202	14(42.4%)	19(57.6%)	5.690	0.017*
T3-T4	67	30(44.8%)	37(55.2%)			12(17.9%)	55(82.1%)		
Vascular invasion									
Absent	30	22(73.3%)	8(26.7%)	8.048	0.005*	17(56.7%)	13(43.3%)	18.733	0.000*
Present	70	28(40.0%)	42(60.0%)			9(12.9%)	61(87.1%)		
Nodal status									
pN -	52	37(71.2%)	15(28.8%)	16.058	0.000*	23(44.2%)	29(55.8%)	18.714	0.000*
pN +	48	13(27.1%)	35(72.9%)			3(6.3%)	44(93.8%)		
Clinical stage									
I-II	63	43(68.3%)	20(31.7%)	20.764	0.000*	25(39.7%)	38(60.3%)	16.568	0.000*
III-IV	37	7(18.9%)	30(81.1%)			1(2.7%)	36(97.3%)		

Note: pN^-: no lymph node metastasis; pN^+: node metastasis. $P < 0.05$.

To assess the association of HIF-1α expression with the prognosis of Kazakh ESCC, Kaplan–Meier survival analysis was performed. A significant difference in the survival curves was observed, and higher HIF-1α expression in Kazakh ESCC predicted low overall survival (χ^2 = 8.121, P = 0.004, log-rank test, Fig. 2A). Furthermore, patients with HIF-1α overexpression had worse overall survival and were at greater risk of death after surgery than patients with negative or weak HIF-1α expression (P = 0.004, Fig. 2B)

In addition, univariate survival analysis indicated that high levels of HIF-1α expression, lymph node metastasis, and TNM stage were associated with poor overall survival. Multivariate analysis incorporating all the statistically significant prognostic factors in the univariate analysis demonstrated that HIF-1α overexpression and lymph node metastasis were both independent prognostic indicators (all P< 0.05, Table 4). Together, these data indicate that overexpression of HIF-1α may a potential prognostic biomarker of poor overall survival for patients with ESCC.

Table 4
Univariate and multivariate Cox regression analyses of the prognostic variables in ESCC patients

Variables	Univariate analysis				Multivariate analysis			
	HR	95%CI		P value	HR	95%CI		P value
HIF-1a expression	1.781	1.265	2.508	0.001*	1.481	1.007	2.178	0.046*
Sex	1.220	0.699	2.128	0.485	1.621	0.893	2.943	0.112
Age (> 59)	0.616	0.355	1.070	0.086	0.688	0.394	1.203	0.190
Differentiation	1.014	0.678	1.517	0.945	0.811	0.516	1.275	0.364
Depth of invasion	1.654	0.925	2.957	0.090	1.583	0.832	3.012	0.162
Lymph node metastasis	2.978	1.641	5.404	0.000*	2.441	1.083	5.499	0.031*
TNM stage(III-IV)	2.554	1.442	4.522	0.001*	1.006	0.434	2.332	0.989
Note: Significant difference that 95% CI of HR was not including; HR: hazard radio; CI: confidence interval; P< 0.05.								

Evaluating VEGF expression in Kazakh ESCCs and CANs, and exploring its relationship with clinicopathological features of ESCC

Analysis of the Oncomine esophagus sample data revealed that expression of HIF-1α is closely associated with expression of VEGF mRNA (Fig. 3A-B) in ESCC. Furthermore, overexpression of VEGF mRNA is also associated with ESCC occurrence (Fig. 4E). These data suggest that HIF-1α may promote the occurrence and progression of esophageal cancer through VEGF.

In this study, we observed VEGF staining mostly in cell membranes and cytoplasm (Fig. 4A-4D), and VEGF staining was most prominent in tumor stroma cells, including macrophages and endothelial cells. Based on the VEGF IHC scoring, the expression of VEGF protein in ESCC tissues was significantly higher than in CAN tissues (P< 0.001, Fig. 4F). In order to compare the relationship between VEGF expression and clinical parameters of ESCC, we divided the cases into low VEGF expression (−/1+) and high VEGF expression groups (2+/3+). VEGF expression levels significantly higher in males than in females (85.9% vs. 52.8%, P = 0.001). Cases with high VEGF expression had higher invasiveness, including the depth of invasion (T3-T4 vs. T1-T2 = 82.1% vs. 57.6%, P = 0.017), vascular invasion (present vs. absent = 87.1% vs. 43.3%, P< 0.001), lymph node metastasis of Kazakh ESCC (pN^+ vs. pN^- = 93.8% vs. 55.8%, P< 0.001), and advanced ESCC clinical progress (III–IV vs. I–II = 97.3% vs. 60.3%, P< 0.001) (Table 3).

Distributions of TAMs in Kazakh ESCC, and the correlation between HIF-1α expression, the density of TAMs, and the expression of VEGF in Kazakh ESCC

Macrophage aggregation in hypoxic microenvironments and HIF-1α expression may play a synergistic role in promoting the progression of Kazakh ESCC. To explore this possibility, we used CD163, a marker of TAMs, to evaluate TAM distribution (Fig. 5A-5D). We found the density of TAMs in Kazakh ESCC tumor nests (approximately 15/HPF, 0–45) and stroma (approximately 58/HPF, 9–139) were significantly higher than in CAN epithelia (approximately 2/HPF, 0–10) and stroma (approximately 19/HPF, 3–54) (all $P < 0.001$, Fig. 5F and 5G).

Oncomine data analysis showed that overexpression of CD163 was closely related with occurrence of ESCC (Fig. 5E), and that expression of HIF-1α was positively correlated not only with expression of VEGF, but also with expression of CD163 macrophages (Fig. 3C). In this study, Spearman correlation analysis was used to analyze the relationship between these three factors. Interestingly, we found that HIF-1α expression positively correlated with the amount of CD163-positive TAMs density in the tumor stroma. ($r = 0.266$, $P < 0.05$). The expression of VEGF was also positively correlated with the HIF-1α expression ($r = 0.221$, $P = 0.027$) and the distribution of CD163-positive macrophages ($r = 0.363$, $P < 0.001$). As the correlation between the expression of VEGF and TAMs is stronger than HIF-1α and TAMs ($r = 0.363$ vs. $r = 0.221$, respectively), the regulation of HIF-1α on the expression of VEGF in ESCCs may be mediated through regulation and recruitment of TAMs (Table 5).

Table 5
Cross correlation analyses reveal strong relationships among density of TAM in tumor nest, tumor stroma and the expression of HIF-1 and VEGF in Kazakh ESCCs

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Characteristics	HIF-1α	TAM density in tumor nest	TAM density in tumor stroma	VEGF
HIF-1α	1	0.161	0.266**	0.221*
TAM density in tumor nest	0.161	1	0.481**	0.177
TAM density in tumor stroma	0.266**	0.481**	1	0.363**
VEGF	0.221*	0.177	0.363**	1

Note: ESCCs: Esophageal squamous cell carcinoma tissues. The numbers shown in the table are correlation coefficient r values. Spearman rank correlation analysis was used. $P < 0.05$.

Discussion

Hypoxia is a common characteristic of the tumor microenvironment that occurs in the early stages of tumor development and promotes tumor progression [15]. The transcription factor hypoxia-inducible factor-1 (HIF-1), a dimeric transcription factor encompassing a HIF-1α and a HIF-1β subunit, plays a pivotal role in this process. HIF-1α is the foremost control switch for low oxygen response, and boosts the transcription of downstream genes required for tumor growth, angiogenesis, and metastasis [16]. In breast cancer and renal cancer [17,18], high expression of HIF-1α is closely related to VEGF expression and angiogenesis, which promotes tumor invasion and migration. These data suggest that HIF-1α promotes cancer through VEGF.

In the hypoxic tumor microenvironment, there are a large number of macrophages. Macrophages can undergo phenotypic transformation due to tumor microenvironmental queues to exhibit an M2 polarization phenotype, and then promote tumor progression by enhancing angiogenesis and metastasis [19]. Macrophages preferentially cluster in hypoxic/necrotic areas of the tumor microenvironment and their presence in large numbers is strongly correlated with poor clinical outcomes [20,21]. In previous studies we have demonstrated that macrophages are involved in the occurrence and progression of ESCC; this may be mediated by macrophages promoting the expression of VEGF [22]. Whether HIF-1α is involved in the regulation of M2

phenotype TAMs, and whether it promotes the invasion and metastasis of Kazakh ESCCs by regulating VEGF expression, remains unclear.

In this study, we found that both HIF-1α and VEGF expression in Kazakh ESCC was significantly higher than in CANs (both $P < 0.05$). Furthermore, overexpression of HIF-1α and VEGF were both significantly associated with vascular invasion, lymph node metastasis, and clinical stage (all $P < 0.05$). Moreover, HIF-1α expression was positively correlated with VEGF expression in Kazakh ESCC. These results are consistent with those from previous esophageal carcinoma studies[7,23], and suggest that HIF-1α promotes ESCC through VEGF. We further analyzed the prognostic significance of HIF-1α protein expression in patients with ESCC. We found that patients with higher HIF-1α expression in Kazakh ESCC tissues had poorer prognosis than those with lower HIF-1α expression. These data are similar to reports in other solid tumors, including breast cancer [20] and hepatocellular carcinoma [21]. Univariate survival analysis revealed that high levels of HIF-1α, lymph node metastasis, and TNM stage correlated with shorter overall survival. However, in multiple regression analysis, only HIF-1α expression and lymph node metastasis remained as independent variables significantly associated with poor overall survival. These results agree with reports in non-small cell lung cancer and colorectal cancer [24,25]. Recent reports indicate that HIF-1α staining is prominent at the advancing tumor border rather than in the central regions. Furthermore, stromal cells, such as immune and inflammatory cells, can also exhibit different levels of expression of HIF-1α, especially macrophages [5,26]. These observations indicated that HIF-1α may play an essential role in tumor progression, possibly through the regulation of macrophages.

CD163 as a marker of M2 TAMs to evaluate the distribution of M2 TAMs in Kazakh ESCCs. We found that the density of TAMs in the Kazakh ESCC tumor stroma and nest was significantly higher than in CAN tissues (both $P < 0.05$). At the same time, we observed that the density of CD163-positive TAMs in tumor stroma was positively associated with HIF-1α expression. These data further indicate that HIF-1α may play a role in promoting the progress of tumors through regulating TAMs.

TAMs can facilitate tumor progression through a variety of mechanisms, including promoting of angiogenesis, lymphangiogenesis, matrix remodeling, and suppressing adaptive immunity [27]. These pro-tumor and pro-angiogenesis phenotypes are generally thought to be associated with VEGF secreted by TAMs. Other studies have demonstrated that VEGF plays an important role in the recruitment and polarization of macrophages in tumors, in the induction of macrophages to the M2 TAM phenotype, and in tumor metastasis and progression [28]. To better understand how TAMs and VEGF are connected in ESCC, we assessed the expression of VEGF in Kazakh ESCCs and CANs. We found that VEGF was mainly expressed in tumor stromal cells, including TAMs. Only a small amount of VEGF expression was seen in tumor cells, which is similar with previous findings from models of hepatocellular carcinoma [29]. We found a significant correlation between high density of TAMs and overexpression of VEGF ($P < 0.001$). These results indicate that VEGF may be mainly secreted by tumor stromal cells, especially by TAMs. These results are in agreement with previous studies on esophageal carcinoma [30], and illustrate that TAMs may promote angiogenesis, invasion, and metastasis of ESCC by regulating VEGF expression.

HIF-1α participates in the regulation of VEGF in the hypoxic tumor microenvironment, which promotes the occurrence and development of tumors. Our study shows that HIF-1α and TAMs play synergistic roles in the hypoxic tumor microenvironment. TAMs present in the tumor stroma express more VEGF than cancer cells, and there are also synergistic interactions between TAMs and VEGF in promoting tumor progression and metastasis. We found that the correlation between VEGF expression and TAMs in Kazakh ESCCs is significantly higher than that between VEGF and HIF-1α ($P < 0.001$ vs. $P = 0.027$, Table 4). These data suggest that HIF-1α may promote angiogenesis, invasion, and metastasis of ESCCs through the regulation of VEGF, and that this process is mediated through M2 TAMs, rather than through direct regulation of VEGF by HIF-1α.

Conclusions

Our study shows that overexpression of HIF-1α may increase the distribution of M2 TAMs in Kazakh ESCC, and promote the expression of VEGF, a primary driver of angiogenesis that also plays an important role in the invasion and metastasis of Kazakh ESCC.

Abbreviations

CANs: Cancer adjacent normal tissues; ESCC:Esophageal squamous cell carcinoma; HIF-1α:Hypoxia-induced factor-1α; VEGF:vascular endothelial growth factor; TAM:tumor associated macrophage; OS:overall survival

Declarations

Availability of data and materials

Data and materials will be shared.

Ethics approval and consent to participate

This work was approved by the Medical Ethics Committee of First Affiliated Hospital of Shihezi University Medical College (approval number: 2018-038-01) and the procedures involving human subjects were in accordance with the Declaration of Helsinki and informed consent was obtained from all participants included in the work, in agreement with institutional guidelines.

Consent for publication

Informed consent for publication was obtained from all participants.

Competing interests

Non competing interests.

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Authors' contributions

JXL carried out the experiments, performed the data analysis and writing the manuscript. XYF, JXR helped with data collection, interpretation and statistical analysis, JCH, YX, WXL, LWH, CXB, SXH, GWE assisted in preparing the tissue microarray, immunohistochemical staining and scoring all IHC slides. YL, LCX, PLJ helped with pathological diagnosis. HJM and LF contributed to the design of study and coordination.

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Figures

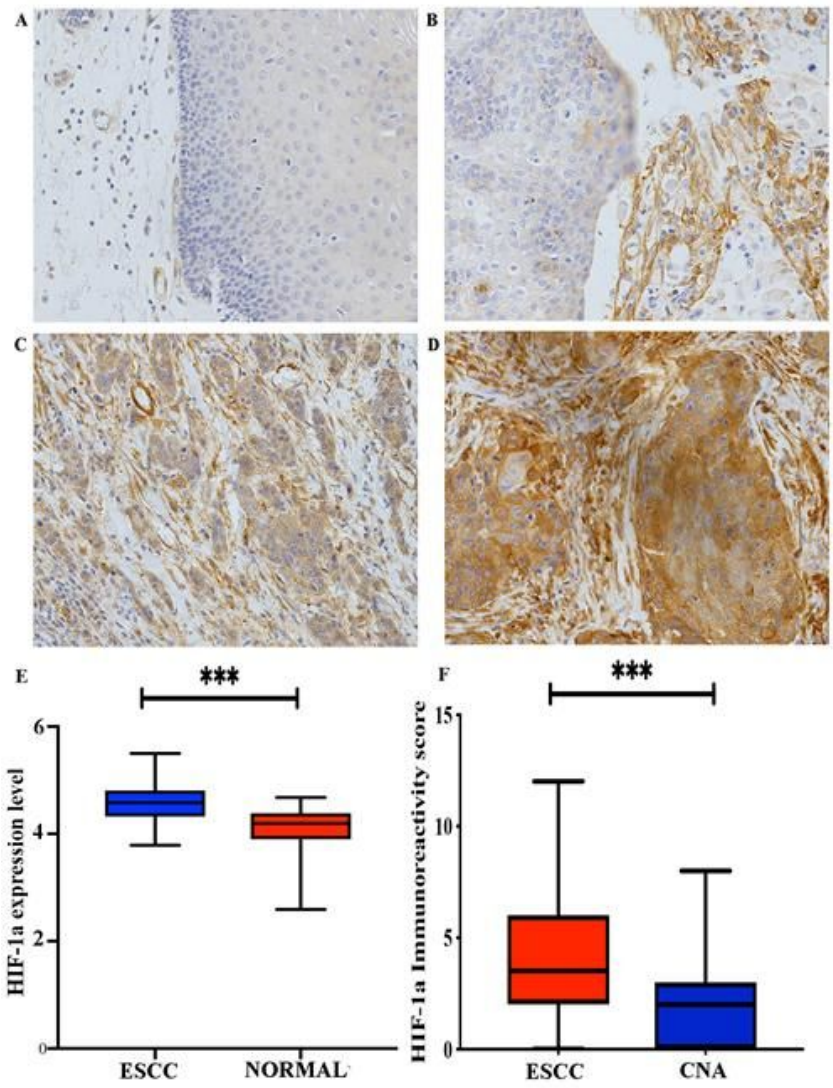

Figure 1

Immunohistochemical staining of HIF-1α in Kazakh ESCC and CAN tissues. HIF-1α staining is primarily observed in tumor stroma (cell membranes and cytoplasm); some ESCC cells also exhibit staining (×200). (A) Negative HIF-1α staining is shown in CAN tissues (scored as 0). (B) Weak HIF-1α staining is shown in CAN tissues (scored as 1). (C) and (D) show moderate and strong HIF-1α staining in Kazakh ESCC tissues (scored as 2 and 3, respectively). (E). Analysis HIF-1α expression from Oncomine (n = 53 samples). HIF-1α was overexpressed in ESCC tissue compared with normal tissue ($P < 0.001$) (F) Boxplot showing that HIF-1α expression levels are significantly higher in ESCC than in CAN tissue from the Kazakh population ($P < 0.001$).

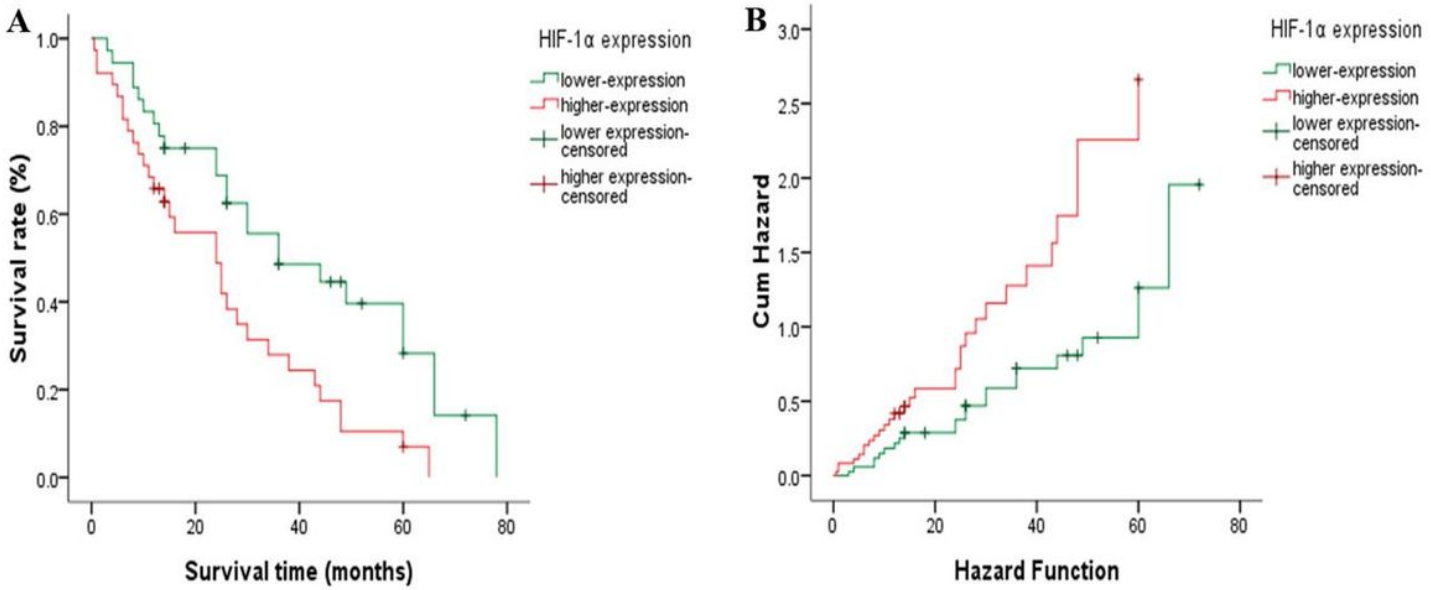

Figure 2

Overexpression of HIF-1α is closely related with poor prognosis of Kazakh ESCC patients. (A) Kazakh ESCC patients with overexpression of HIF-1α experienced significantly shorter survival period after surgery than those with low HIF-1α levels (P < 0.05). (B) Patients with overexpression of HIF-1α had a greater risk of death than those with lower HIF-1α levels (P < 0.05).

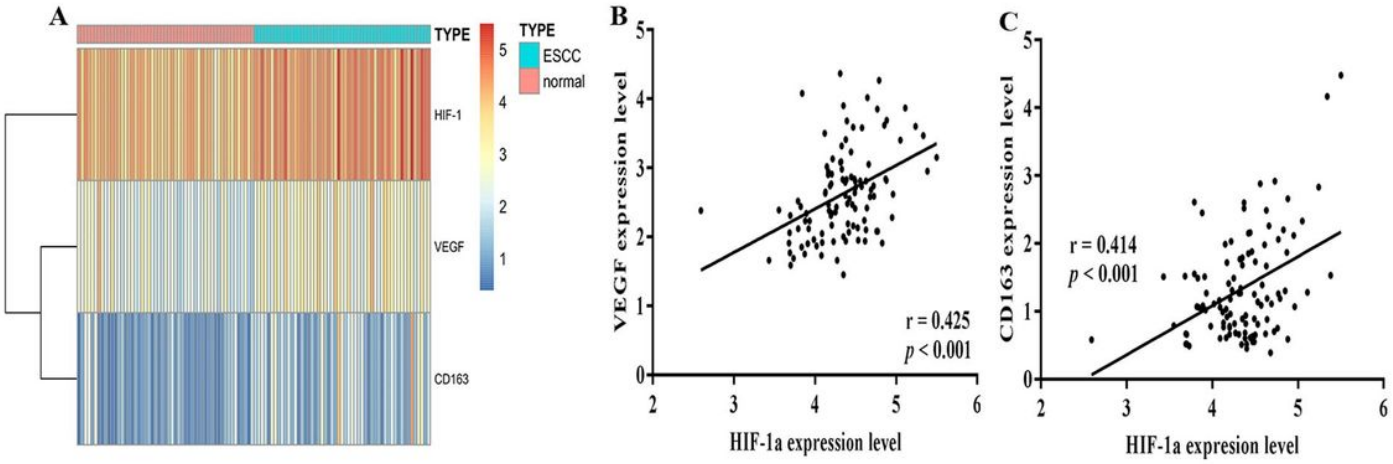

Figure 3

The expression of HIF-1α, VEGF, and CD163 was compared between ESCC and normal esophagus tissues, and correlations and interactions between the HIF-1α, VEGF, and CD163 were explored by Oncomine. (A) Heatmap and clusters of HIF-1α, VEGF, and CD163 expression in 106 samples, including 53 cases (red) and 53 controls (blue) from the Oncomine database. (B) There was a significant positive correlation between HIF-1α and VEGF expression in the esophagus samples from the Oncomine data (r = 0.425, P < 0.001). (C) There was a significant positive correlation between HIF-1α and CD163 expression in the esophagus samples from the Oncomine data (r = 0.414, P < 0.001).

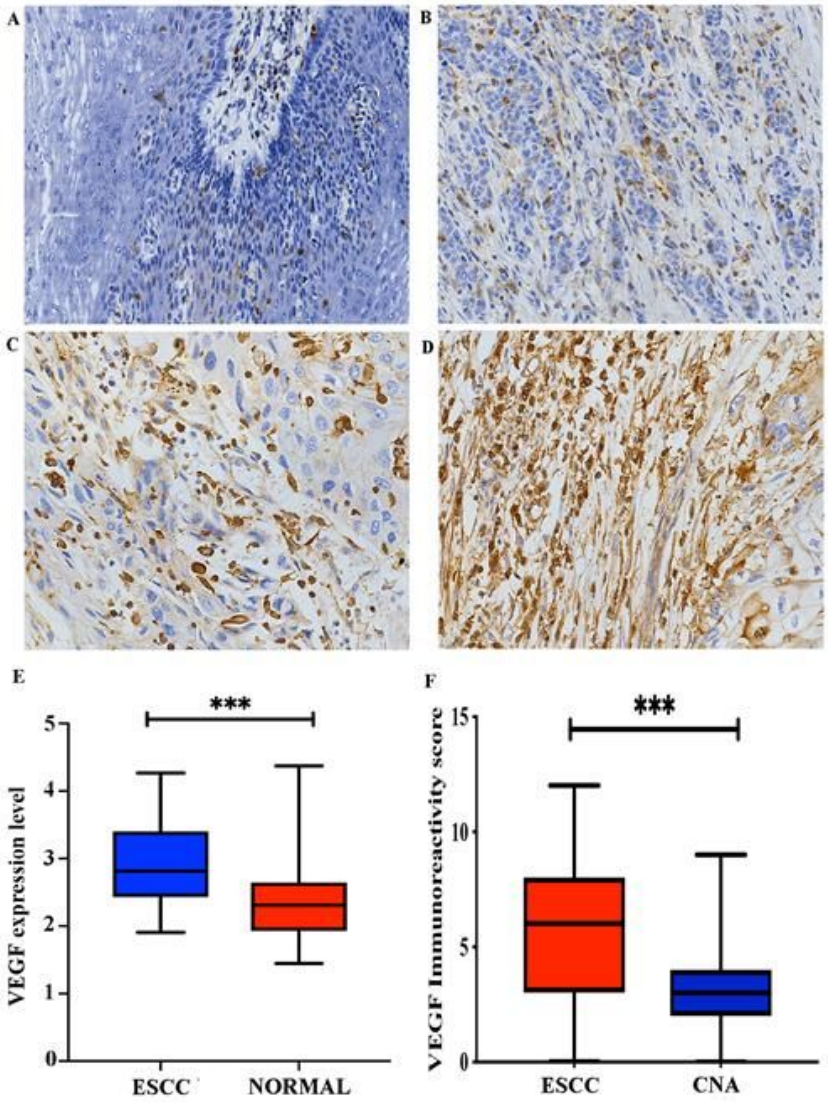

Figure 4

Immunohistochemical staining of VEGF in Kazakh ESCC and CAN tissues. VEGF staining was primarily observed in tumor stroma (cell membranes and cytoplasm); some ESCC cells also exhibit positive staining (×200). (A) Negative VEGF staining was shown in CAN tissues (scored as 0). (B) Weak VEGF staining was shown in ESCC tissues (scored as 1). (C) and (D) show moderate and strong VEGF staining in Kazakh ESCC tissues (scored as 2 and 3, respectively). (E) Analysis of VEGF expression from the Oncomine data (n = 35 samples). VEGF was overexpressed in ESCC tissue compared with the normal tissue ($P < 0.001$). (F) Boxplot showing that VEGF expression levels in ESCC were significantly higher than in CAN tissue from the Kazakh population ($P < 0.001$).

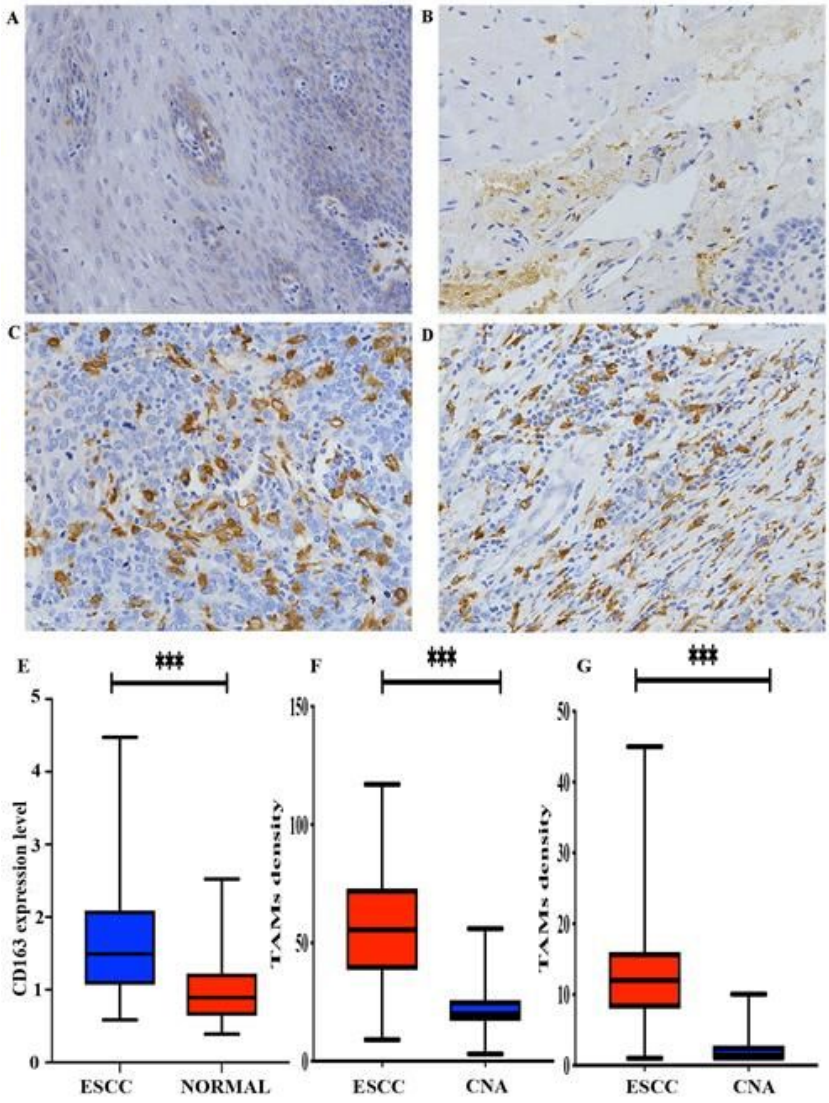

Figure 5

Distribution of CD163-positive TAMs in Kazakh ESCC tumor nest tissue, CAN epithelia, and CAN stroma. (A) and (B) show the density of TAMs in CAN epithelia and stroma, respectively; (C) and (D) show the distribution of TAMs in tumor nest and stroma in ESCC, respectively (×200). (E) Analysis of CD163 expression from the Oncomine data (n = 35 samples). CD163 was overexpressed in ESCC tissue compared with normal tissue ($P < 0.001$). (F) and (G) boxplots demonstrating that the density of CD163-positive TAMs in Kazakh ESCC tumor stroma (F) and tumor nest (G) are significantly higher than in CAN stroma or CAN epithelia (both $P < 0.001$).