Aberrant Cyclin E and Hepatocyte Growth Factor Expression, Microvascular Density, and Micro-Lymphatic Vessel Density in Esophageal Squamous Cell Carcinoma

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Abstract

Cyclin E and hepatocyte growth factor (HGF) have been observed as a multifaceted factor in many cancers, and the assessment of microvascular density (MVD) and micro-lymphatic vessel density (MLVD) has been used to quantify tumor angiogenesis and lymphangiogenesis. The aim of this study was to explore the association between expression of cyclin E, HGF, MVD, and MLVD, and clinicopathologic parameters in esophageal squamous cell carcinoma (ESCC). The expression of cyclin E, HGF, MVD, and MLVD were detected using immunohistochemically anticyclin E, HGF, CD34, and lymphatic vessel endothelial hyaluronan receptor 1 in 168 surgically resected ESCC cases and 30 normal esophageal mucosal samples. The expression levels of cyclin E, HGF, MVD, and MLVD were higher compared to controls. High cyclin E and HGF expression was found more frequently in the tumors larger than 5 cm ($P < .001$), with poorer differentiation ($P = .034$) and higher tumor node metastasis (TNM) staging ($P = .009$) compared to their counterparts. Both MVD and MLVD values were found to be higher in the tumors larger than 5 cm ($P < .001$), with poorer differentiation ($P < .001$) and higher TNM staging ($P < .001$) compared to their counterparts. Furthermore, the expression of MVD and MLVD in both the high cyclin E and high HGF expression groups was significantly higher compared to the low cyclin E and HGF expression groups ($P < .001$). This study demonstrated that high cyclin E and HGF expression is closely correlated with tumor size, tumor differentiation degree, and TNM stage in patients with ESCC. These findings proposed that cyclin E and HGF could serve as novel molecular markers for preoperational evaluation of ESCC.

Keywords

cyclin E, hepatocyte growth factor, microvascular density, micro-lymphatic vessel density, immunohistochemistry

Introduction

Esophageal cancer (EC) is one of the most common and deadly malignancies in the world, causing over 400 000 deaths every year[1] Although the incidence of EC in China is decreasing, the mortality rate is still high.[2] Esophageal squamous cell carcinoma (ESCC) accounts for more than 90% of EC, followed by esophageal adenocarcinoma. Currently, multiple factors and complex biology, including lymphatics and blood vessel growth, are involved in occurrence and

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development of ESCC. Despite significant advances in EC treatment, the overall 5-year-survival rate is only between 5% and 20%.[3]

Cyclin E is an important positive regulatory factor involved in the regulation of the G1/S cell cycle transition.[4] Cyclin E is overexpressed in a variety of malignant tumors and is closely related to the degree of malignancy and disease prognosis. Accumulating evidence has demonstrated that cyclin E is oncogenic and can be an early diagnostic and prognostic biomarker.[5,6] Hepatocyte growth factor (HGF) was first isolated from the serum of rats that had undergone a partial hepatectomy. Hepatocyte growth factor is a strong mitogen that stimulates hepatocyte proliferation.[7,8]

In the present study, we performed immunohistochemical staining to detect microvascular density (MVD), micro-lymphatic vessel density (MLVD), and expression of cyclin E and HGF in ESCC and adjacent normal esophageal tissue. We also analyzed the association between expression of cyclin E, HGF, MVD, and MLVD in tumors and in clinicopathologic parameters of patients with ESCC. Our data revealed the potential clinical value of using cyclin E and HGF as indicators of invasion and metastasis in ESCC.

Materials and Methods

Patients and Specimens

A total of 168 surgically resected ESCC samples and 30 normal control esophageal mucosal samples (taken 5.0 cm away from the tumor edge) were collected. None of the patients received chemotherapy, radiotherapy, or immunotherapy prior to surgery. All tissue specimens were obtained from the First Affiliated Hospital of Bengbu Medical College in Bengbu, Anhui Province, China, between September 2016 and August 2018. The pathology of all tissue was histologically confirmed. This study was approved by the Institutional Review Board of The First Affiliated Hospital of Bengbu Medical College. All patients provided informed consent to participate in the study. For the ESCC samples, 96 samples were from males and 72 samples were from females. There were 112 samples from patients older than 60 years and 56 samples from patients younger than 60 years. Twenty-two samples were from the upper segment of the esophagus, 92 samples were from middle segment, and 54 samples were from the lower segment of the esophagus. There were 78 cases of medullary, 52 cases of ulcerative, 29 cases of mushroom, and 9 cases of sclerotic type. Tumor sizes ranged from 1.5 to 6.8 cm. Seventy-two tumors were larger than 5.0 cm, and 96 tumors were smaller than 5.0 cm in diameter. There were 10 well-differentiated cases, 110 mid-differentiated cases, and 48 poorly differentiated cases. According to the tumor node metastasis (TNM) staging system of the Union for International Cancer Control, 28 cases were stage I, 92 cases were stage II, and 48 cases were stage III.

Immunohistochemistry

All samples were fixed in 10% buffered formalin, embedded in paraffin, and cut into serial sections (5 μm). Protein expression of cyclin E, HGF, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), and CD34 were detected by immunohistochemistry. Briefly, all slides were deparaffinized, dehydrated, and washed for 10 minutes in phosphate buffered saline (PBS). To deactivate endogenous peroxidases, tissue sections were incubated with 0.3% hydrogen peroxide at room temperature for 20 minutes and soaked in PBS for 3 minutes. The sections were blocked with goat serum and then incubated with mouse monoclonal antibodies against cyclin E (dilution: 1:200, catalog no. ab3927; Abcam, Shanghai), HGF (dilution: 1:200, catalog no. ab24865; Abcam, Shanghai), CD34 (dilution: 1:200, catalog no. ab762; Abcam, Shanghai), and LYVE-1 (dilution: 1:200, catalog no. ab14917; Abcam, Shanghai) overnight at 4°C. After washing with PBS, the tissue sections were incubated with secondary antibodies, and signal was developed using diaminobenzidine and hematoxylin counterstaining.

Immunostaining Evaluation and Quantitative Measurement of MVD and MLVD

All stained samples were scored blindly by 2 pathologists. Over 100 cells from 5 random fields of view for each sample were scored. The score was based on the proportion of positively stained tumor cells. A score of 0 denoted no positive cells were observed. A score of 1 indicated that <10% of cells were positive. A score of 2 meant that 10% to 35% of cells were positive. A score of 3 indicated that 35% to 75% of cells were positive. A score of 4 denoted that more than 75% of cells were positive. Staining intensity was divided into 4 categories: 0 denoted absence of staining, 1 signified the presence of a pale-yellow hue, 2 represented the presence of a yellow or dark-yellow hue, and 3 represented the presence of a brown or tan color. The final score was calculated by multiplying the percentage of positive and the staining intensity scores. A final score ≥4 was considered high expression and a final score <4 was considered low expression.

Both MVD and MLVD were evaluated by 2 experienced pathologists who were blinded to the experimental conditions. CD34 immunostaining was used to visualize microvascular endothelial cells, while LYVE-1 was used to visualize micro-lymphocytic endothelial cells. The pathologists reviewed the sections microscopically under 100× magnification and subsequently selected 5 random microscopic fields at 400× magnification to capture images. Both MVD and MLVD were calculated using the average number of microvessels and micro-lymphatic vessels, respectively, from five 400× microscopic fields.

Statistical Analysis

All statistical analyses were performed using SPSS software version 20.0. All data were analyzed using the Student *t* test.

Table 1. Expression of Cyclin E and HGF in ESCC and Control Tissues.

	Cyclin E				HGF			
Variable	High Expression	Low Expression	χ^2	P	High Expression	Low Expression	χ^2	P
ESCC	97	71	14.524	<.001	92	76	4.680	.031
Control	6	24			10	20		

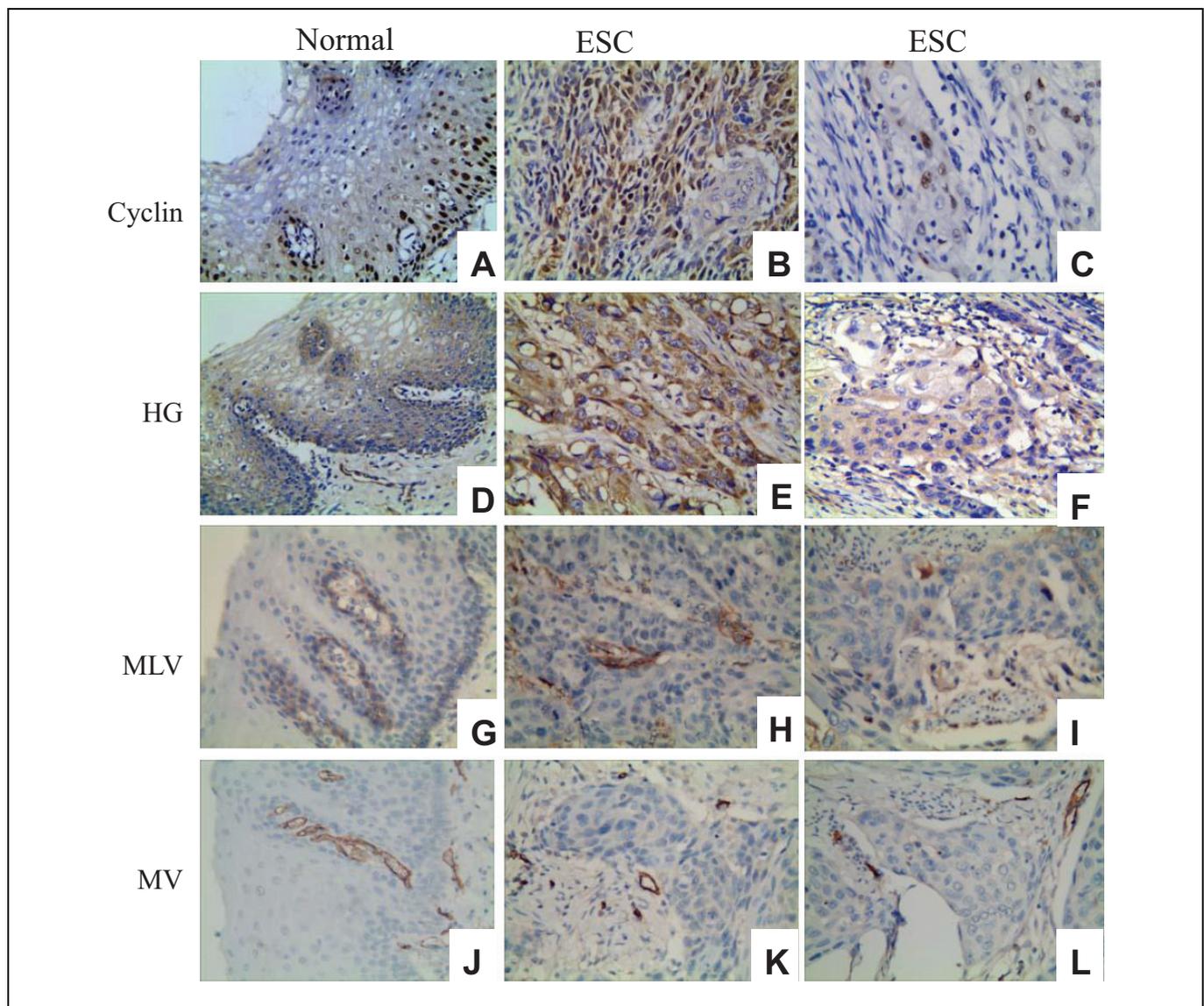Abbreviations: ESCC, esophageal squamous cell carcinoma; HGF, hepatocyte growth factor expression.

Figure 1. Expression of cyclin E and HGF in ESCC and control tissue (Elivision, ×400). Positive staining for cyclin E in the nucleus of the normal cells (A). High expression (B) and low expression (C) for cyclin E in the ESCC tumor cells. Positive staining for HGF in the cytoplasm of the normal cell (D). High expression (E) and low expression (F) for HGF in the ESCC tumor cells. Staining for MLVD in normal tissue (G). High expression (H) and low expression (I) for MLVD in the ESCC tumor tissue. Staining for MVD in normal tissue (J). High expression (K) and low expression (L) for MVD in the ESCC tumor tissue. HGF indicates hepatocyte growth factor expression; MVD, microvascular density, MLVD, micro-lymphatic vessel density; ESCC, esophageal squamous cell carcinoma.

A $P < .05$ was considered statistically significant. Counted data were presented as frequency and percentage and analyzed with a χ^2 analysis or Fisher exact test. Measurement data were presented as the mean/median $\pm$ standard deviation. Data with a normal distribution were analyzed by an F test or t test.

Table 2. Association Between Cyclin E, HGF Expression, and Clinicopathologic Characteristics of Patients With ESCC.

	Cyclin E				HGF			
Variable	High Expression	Low Expression	χ^2	P	High Expression	Low Expression	χ^2	P
Gender								
Male	55	41	0.018	.892	51	45	0.242	.623
Female	42	30			41	31		
Age, years								
≥60	63	49	0.305	.581	57	55	2.030	.154
<60	34	22			35	21		
Tumor location								
Upper	12	10	0.129	.937	15	7	2.115	.347
Middle	54	38			47	45		
Lower	31	23			30	24		
Gross type								
Medullary type	45	33	1.780	.619	40	38	1.539	.673
Ulcer type	33	19			28	24		
Mushroom type	14	15			18	11		
Sclerotic type	5	4			6	3		
Tumor size								
≥5.0 cm	55	17	17.962	<.001	49	23	8.988	<.001
<5.0 cm	42	54			43	53		
Differentiation agree								
Well	2	8	6.77	.034	3	7	10.289	.006
Middle	64	46			54	56		
Poor	31	17			35	13		
TNM staging								
I	9	19	9.455	.009	8	20	18.994	<.001
II	56	36			51	41		
III	32	16			38	10		

Abbreviations: ESCC, esophageal squamous cell carcinoma; HGF, hepatocyte growth factor expression; TNM, tumor node metastasis.
[a]n = 168.

Results

Cyclin E and HGF Expression in ESCC and Control Tissue

High cyclin E expression was detected in 57.74% of ESCC samples and in 20.00% of controls ($P < .001$; Table 1). High HGF expression was observed in 54.76% of ESCC samples and in 33.33% of controls ($P = .031$; Table 1). The staining results showed that cyclin E was mainly localized in the nucleus in both control cells (Figure 1A) and ESCC (Figure 1B) cells, and HGF mainly was expressed in the cytoplasm in both control cells (Figure 1D) and ESCC (Figure 1E) cells.

Association of Cyclin E and HGF Expression With Clinicopathologic Characteristics of Patients With ESCC

Cyclin E expression did not correlate with gender ($P = .892$), age ($P = .581$), tumor location ($P = .937$), or gross type ($P = .619$; Table 2), but it did closely correlate with tumor size ($P < .001$), tumor differentiation degree ($P = .034$), and TNM stage ($P = .009$). Expression of HGF was closely correlated with tumor size ($P < .001$), tumor differentiation degree ($P = .006$), and TNM stage ($P < .001$), but it did not correlate with

gender ($P = .623$), age ($P = .154$), tumor location ($P = .347$), or gross type ($P = .673$; Table 2).

Microvascular Density and MLVD Expression in ESCC and Control Tissues

CD34 immunostaining was used to measure MVD, while LYVE-1 was used to measure MLVD. The staining patterns of CD34 and LYVE-1 appeared as channel-like structures in both the control (Figure 1J and G) and the ESCC specimens (Figure 1K and H). The results showed that MVD ($P < .001$) and MLVD ($P < .001$) levels in ESCC samples were significantly higher than that in the controls (Table 3).

Association Between MVD and MLVD Levels With Clinicopathologic Characteristics of Patients With ESCC

Levels of MVD were positively correlated with tumor size ($P < .001$), tumor differentiation degree ($P < .001$), and TNM stage ($P < .001$) but were not correlated with gender ($P = .068$), age ($P = .221$), tumor location ($P = .800$), or gross type ($P = .949$; Table 4). Levels of MLVD were significantly correlated with tumor size ($P = .003$), tumor differentiation degree ($P < .001$), and TNM stage ($P < .001$)

Table 3. MVD and MLVD in ESCC and Control Tissues.

		MVD				MLVD		
Variable	n	Value (Mean ± SD)	t	P	n	Value (Mean ± SD)	t	P
ESCC	168	9.43 ± 2.98	14.302	<.001	168	8.27 ± 1.95	18.127	<.001
Control	30	1.58 ± 0.83			30	1.76 ± 0.54		

Abbreviations: ESCC, esophageal squamous cell carcinoma; MVD, microvascular density, MLVD, micro-lymphatic vessel density; SD, standard deviation.

Table 4. Association Between MVD, MLVD, and Clinicopathologic Characteristics of Patients With ESCC .[a]

		MVD				MLVD		
Variable	n	Value (Mean ± SD)	t/F	P	n	Value (Mean ± SD)	t/F	P
Gender								
Male	96	9.68 ± 2.07	1.839	.068	96	8.13 ± 1.76	1.621	.107
Female	72	9.14 ± 1.60			72	8.50 ± 0.93		
Age, years								
≥60	112	9.25 ± 1.59	1.229	.221	112	8.39 ± 1.07	1.728	.089
<60	56	9.60 ± 2.01			56	8.09 ± 0.94		
Tumor location								
Upper	22	9.26 ± 1.65	0.22	.800	22	8.10 ± 0.99	0.42	.657
Middle	92	9.50 ± 1.74			92	8.32 ± 1.43		
Lower	54	9.31 ± 2.47			54	8.15 ± 1.22		
Gross type								
Medullary type	78	9.63 ± 2.17	0.12	.949	78	8.48 ± 1.86	0.89	.446
Ulcer type	52	9.42 ± 2.07			52	8.01 ± 1.65		
Mushroom type	29	9.55 ± 1.89			29	8.37 ± 1.23		
Sclerotic type	9	9.38 ± 2.86			9	8.09 ± 0.96		
Tumor size								
≥5.0 cm	72	9.93 ± 2.83	6.015	<.001	72	8.92 ± 1.64	3.035	.003
<5.0 cm	96	7.86 ± 1.59			96	8.08 ± 1.87		
Differentiation agree								
Well	10	6.52 ± 2.04	15.75	<.001	10	5.37 ± 0.85	41.68	<.001
Middle	110	8.79 ± 2.97			110	7.71 ± 1.92		
Poor	48	11.03 ± 2.56			48	10.03 ± 1.60		
TNM staging								
I	28	7.23 ± 2.10	29.50	<.001	28	6.10 ± 1.01	88.86	<.001
II	92	9.01 ± 2.48			92	7.92 ± 1.24		
III	48	11.76 ± 3.17			48	10.56 ± 2.05		

Abbreviations: ESCC, esophageal squamous cell carcinoma; HGF, hepatocyte growth factor expression; MLVD, micro-lymphatic vessel density; MVD, micro-vascular density, TNM, tumor node metastasis SD, standard deviation.
[a]n = 168.

but were not correlated with gender ($P = .107$), age ($P = .089$), tumor location ($P = .657$), or gross type ($P = .446$; Table 4).

Association Between Cyclin E and HGF Expression With MVD and MLVD Levels in ESCC Tissue

Levels of MVD and MLVD in the high cyclin E expression group were significantly higher compared to the low cyclin E expression group ($P < .001$; Table 5). Moreover, MVD and MLVD levels in the high HGF expression group were also significantly higher compared to the low HGF expression group ($P < .001$; Table 5).

Discussion

Despite significant advances in ESCC detection capabilities, early diagnostic rates remain low. Compared to early ESCC, the prognosis of advanced ESCC is very poor. Early diagnosis and treatment of ESCC is key to a favorable prognosis. Therefore, exploring accurate biomarkers to improve early diagnosis and prognosis of ESCC is of great importance. In the current study, we examined expression of cyclin E and HGF in ESCC

Table 5. Association Between Cyclin E, MVD, and MLVD in ESCC Tissue.

Variable	n	MVD	t	P	MLVD	t	P
Cyclin E high expression	97	11.44 ± 2.67	12.085	<.001	10.23 ± 1.96	16.657	<.001
Cyclin E low expression	71	7.03 ± 1.78			5.93 ± 1.10		
HGF high expression	92	10.20 ± 2.21	4.945	<.001	8.90 ± 1.73	5.463	<.001
HGF low expression	76	8.58 ± 1.99			7.53 ± 1.47		

Abbreviations: ESCC, esophageal squamous cell carcinoma; HGF, hepatocyte growth factor expression; MLVD, micro-lymphatic vessel density; MVD, micro-vascular density.

and normal esophageal mucosal tissue and analyzed the association between cyclin E and HGF expression with clinicopathologic characteristics of patients with ESCC. Our results demonstrated that cyclin E and HGF were expressed in both ESCC and normal esophageal mucosal tissue. However, cyclin E expression was high in 57.74% of cases with ESCC and 20% of controls, while HGF expression was high in 54.76% of cases with ESCC and in 33.33% of controls. The present study demonstrated that the expression of cyclin E and HGF in ESCC was significantly upregulated compared to that in normal esophageal mucosal tissue.

Abnormal cell cycle regulation and uncontrolled cell proliferation are important molecular mechanisms underlying tumorigenesis.[9,10] As a regulator of S phase, cyclin E plays a critical role in the regulation of tumor progression. It has been reported that patients with high cyclin E expression tend to be at more advanced TNM stages than those with low cyclin E expression, suggesting that high cyclin E expression is significantly associated with high tumor proliferation and may be involved in ESCC progression.

Hepatocyte growth factor, a multifunctional cytokine, is involved in the signal transduction cascade of the HGF-c-met system, which can stimulate proliferation; dedifferentiation of various cell types; weaken cell-to-cell adhesion interactions; and promote the migration and invasion of tumor cells and the formation of tumor vessels.[11,12] Previous studies have shown that cyclin E and HGF are highly expressed in multiple malignant tumor types.[13-16] In the present study, the high expression of cyclin E and HGF was significantly associated with tumor size, differentiation degree, and TNM stage in patients with ESCC. These data suggest that the high expression of cyclin E and HGF plays an important role in the development of ESCC and may be one of the mechanisms underlying invasion and metastasis of ESCC. The results were consistent with those of many previous studies.[17-19]

The MVD is widely used to evaluate tumor angiogenesis. An increasing amount of clinical and experimental evidence has shown that with an increase in MVD, the tumor undergoes invasion and metastasis. The MLVD can reflect the microenvironmental status of tumors. The microenvironment of tumors affects the biological behavior of tumor cells including proliferation, infiltration, and metastasis. In the present study, MVD and MLVD levels in ESCC samples were significantly higher than that in the controls. Moreover, MVD and MLVD levels were closely correlated with tumor size, tumor differentiation degree, and TNM stage. These findings were coincident with

previous reports.[20-22] These data also suggest that angiogenesis and lymphangiogenesis in tumor tissue contribute to tumor proliferation, invasion, and metastasis. Furthermore, there is a positive association between MVD, MLVD, and cyclin E and HGF expression, indicating that cyclin E and HGF may contribute to angiogenesis and lymphangiogenesis of ESCC. These data suggest that high cyclin E and HGF expression may be involved in angiogenesis and lymphangiogenesis of ESCC and play a role in the proliferation, invasion, and metastasis of ESCC.

There are some limitations in the current study. The current study only investigated the relationship between cyclin E, HGF, MVD, and MLVD and clinicopathologic characteristics of patients with ESCC. The potential mechanisms detailing this biology have not been determined, and more work is needed to elucidate this biology. In addition, the sample size in the current study is limited. Finally, this study was carried out in our center only, and a multicenter research study is needed to further understand the prevalence of our findings in broader populations.

In conclusion, our study demonstrated that high cyclin E and HGF expression levels were significantly associated with tumor size, tumor differentiation degree, and TNM stage in patients with ESCC and indicates a direct link between MVD and MLVD. These markers might serve as novel biomarkers for preoperational evaluation of ESCC.

Authors' Note

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Declaration of Conflicting Interests

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References

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality world-wide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136(5):e359-e386.

2. Jemal A, Bray F, Center MM, Ferlay J, et al. Global cancer statistics. *CA Cancer J Clin*. 2011;61(2):69-90.

3. Chen W, Zheng R, Baade PD, et al. Cancer statistics in china, 2015. *CA Cancer J Clin*. 2016; 66(2):115-132.

4. Zhou Z, Bandla S, Ye J, Xia Y, et al. Cyclin E involved in early stage carcinogenesis of esophageal adenocarcinoma by SNP DNA microarray and immunohistochemical studies. *BMC Gastroenterol*. 2014;17(14):78.

5. Santala S, Talvensaari-Mattila A, Soini Y, et al. Cyclin E expression correlates with cancer-specific survival in endometrial endometrioid adenocarcinoma. *Anticancer Res*. 2015;35(6): 3393-3397.

6. Luhtala S, Staff S, Tanner M, et al. Cyclin E amplification, over-expression, and relapse-free survival in HER-2-positive primary breast cancer. *Tumour Biol*. 2016;37(7):9813-9823.

7. Tsai PC, Fu YS, Chang LS, et al. Cardiotoxin III inhibits hepatocyte growth factor-induced epithelial-mesenchymal transition and suppresses invasion of MDA-MB-231 cells. *J Biochem Mol Toxicol*. 2016;30(1):12-21.

8. Thayaparan T, Spicer JF, Maher J. The role of the HGF/Met axis in mesothelioma. *Biochem Soc Trans*. 2016;44(2): 363-370.

9. Siu KT, Rosner MR, Minella AC. An integrated view of cyclin E function and regulation. *Cell Cycle*. 2012;11(1):57-64.

10. Duzgun O, Inanc SS. Preoperative CA125 value predicts glisson capsule involvement in patients with peritoneal carcinomatosis undergoing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *Biomark Med*. 2019;13(5): 359-369.

11. Zhang Y, Jain RK, Zhu M. Recent progress and advances in HGF/MET-targeted therapeutic agents for cancer treatment. *Biomed*. 2015;3(1):149-181.

12. Spina A, De Pasquale V, Cerulo G, et al. HGF/c-MET Axis in tumor microenvironment and metastasis formation. *Biomed*. 2015;3(1):71-88.

13. Deng W, Zhou Y, Tiwari AF, et al. p21/Cyclin E pathway modulates anticlastogenic function of bmi-1 in cancer cells. *Int J Cancer*. 2015;136(6):1361-1370.

14. Alsina M, Landolfi S, Aura C, et al. Cyclin E amplification/over-expression is associated with poor prognosis in gastric cancer. *Ann Oncol*. 2015;26(2):438-439.

15. Fajardo-Puerta AB, Mato Prado M, Frampton AE, et al. Gene of the month: HGF. *J Clin Pathol*. 2016;69(7):575-579.

16. Graziano F, Catalano V, Lorenzini P, et al. Clinical impact of the HGF/MET pathway activation in patients with advanced gastric cancer treated with palliative chemotherapy. *Pharmacogenomics J*. 2014;14(5):418-423.

17. Choy B, LaLonde A, Que J, et al. MCM4 and MCM7, potential novel proliferation markers, significantly correlated with Ki-67, Bmi1, and cyclin E expression in esophageal adenocarcinoma, squamous cell carcinoma, and precancerous lesions. *Hum Pathol*. 2016;57:126-135.

18. Matsumoto M, Furihata M, Ohtsuki Y, et al. Immunohistochemical characterization of p57KIP2 expression in human esophageal squamous cell carcinoma. *Anticancer Res*. 2000;20(3B): 1947-1952.

19. Xu Z, Wang S, Wu M, et al. TGFβ1 and HGF protein secretion by esophageal squamous epithelial cells and stromal fibroblasts in oesophageal carcinogenesis. *Oncol Lett*. 2013;6(2):401-406.

20. Kumagai Y, Tachikawa T, Higashi M, et al. Thymidine phosphorylase and angiogenesis in early stage esophageal squamous cell carcinoma. *Esophagus*. 2018;15(1):19-26.

21. Qu W, Jin Dong FU, Yang F, et al. Clinical implications of PTEN and VEGF expression status, as well as microvessel density in esophageal squamous cell carcinoma. *Oncol Lett*. 2015;10(3): 1409-1415.

22. Omoto I, Matsumoto M, Okumura H, et al. Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma. *Oncol Lett*. 2014;7(4):1027-1032.