Clinical and biological significance of stem-like CD133⁺CXCR4⁺ cells in esophageal squamous cell carcinoma

Chunlai Lu, MD,^a Fengkai Xu, MD,^a Jie Gu, MD,^a Yunfeng Yuan, MD,^a Guangyin Zhao, MD,^a Xiaofang Yu, MD,^b and Di Ge, MD^a

ABSTRACT

Objectives: Esophageal squamous cell carcinoma is one of the most frequent malignant tumors. Cancer stem cells are considered to be responsible for tumor growth, metastasis, and recurrence. Cluster of differentiation 133 (CD133) and C-X-C chemokine receptor type 4 (CXCR4) are frequently applied markers for the identification and isolation of cancer stem cells. However, few studies have investigated the coexpression of CD133 and CXCR4 in esophageal squamous cell carcinoma. This study aims to explore the clinical and biological role of stem-like CD133⁺CXCR4⁺ cells in esophageal squamous cell carcinoma.

Methods: Immunohistochemical staining was performed to detect the expression of CD133 and CXCR4 in esophageal squamous cell carcinoma tissues of patients. Flow cytometry and fluorescence-activated cell sorting were applied to analyze and isolate each subgroup in esophageal squamous cell carcinoma cell line TE-1. The characteristic differences between each subgroup were assayed in vitro. The association between CD133/CXCR4 expression and patients' prognosis was analyzed by Kaplan–Meier and Cox regression.

Results: Among 154 patient tissues, concomitant high CD133–CXCR4 expression accounts for 20.78% (32/154). In vitro, CXCR4⁺ cells (CD133⁺CXCR4⁺ and CD133[–]CXCR4⁺) showed high invasive potential and CD133⁺CXCR4⁺ cells showed high proliferative capacity. Clinically, patients with concomitant high CD133–CXCR4 expression had decreased disease-free survival and overall survival ($P < .01$).

Conclusions: Esophageal squamous cell carcinoma cells coexpressing CD133 and CXCR4 possess the characteristics of cancer stem cells. The concomitant high CD133–CXCR4 expression might be a novel marker for predicting the poor prognosis of patients with esophageal squamous cell carcinoma, and CD133 and CXCR4 may serve as potential therapeutic targets. (*J Thorac Cardiovasc Surg* 2015;150:386–95)

DFS and OS curves of 154 enrolled patients with ESCC with different CD133 and CXCR4 expression.

Central Message

Stem-like CD133⁺CXCR4⁺ ESCC cells predict the prognosis of patients.

Perspective

This study demonstrated that ESCC cells coexpressing CD133 and CXCR4 possess the partial characteristics of CSCs (high proliferative and invasive ability). The concomitant high CD133–CXCR4 expression might be a novel marker for predicting the poor prognosis of patients with ESCC, and CD133 and CXCR4 may serve as potential therapeutic targets.

See Editorial Commentary page 396.

Esophageal squamous cell carcinoma (ESCC) is one of the most frequent malignant tumors worldwide. In China, the incidence of ESCC is higher than that of esophageal

adenocarcinoma.¹ Despite recent advances in therapeutic strategies, the 5-year survival remains low because of the difficulties in early diagnosis and the high likelihood for distant metastasis.² A hypothesis was recently presented that a rare population of cells exists in the solid cancer and contributes to treatment resistance and early metastasis. These cells are named “cancer stem cells” (CSCs) or “tumor-initiating cells.”³ CSCs have been viewed as a cell subpopulation within solid tumors that possesses the capacity of self-renewal, differentiation into heterogeneous lineages of cancer cells, and chemotherapy and radiotherapy resistance. Therefore, residual CSCs are considered to lead to therapeutic failure and as a potential target for novel therapies.

Cluster of differentiation 133 (CD133) and C-X-C chemokine receptor type 4 (CXCR4) are frequently applied

From the Departments of ^aThoracic Surgery and ^bNephrology, The Affiliated Zhongshan Hospital of Fudan University, Shanghai, People's Republic of China. This work was supported by the National Natural Science Foundation (81302099) and the Doctoral Fund for Young Scholar of Ministry of Education of China (20110071120065).

CL and FX contributed equally to this work.

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Address for reprints: Di Ge, MD, Department of Thoracic Surgery, The Affiliated Zhongshan Hospital of Fudan University, Shanghai, People's Republic of China 200032 (E-mail: gedi6902@hotmail.com).

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Abbreviations and Acronyms

CSC	= cancer stem cell
CXCR4	= C-X-C chemokine receptor type 4
DFS	= disease-free survival
DMEM	= Dulbecco's Modified Eagle Medium
ESCC	= esophageal squamous cell carcinoma
OS	= overall survival
SDF-1	= stromal cell-derived factor 1

markers for the identification and isolation of CSCs. CD133, also known as prominin-1, is a kind of 5-transmembrane glycoprotein expressed on hematopoietic stem cells and endothelial stem cells. It has been reported to be a marker of CSCs in the brain, colon, pancreas, prostate, liver, lung, kidney, and esophagus.⁴⁻¹² CXCR4 is a membrane-bound receptor that selectively binds to the CXC chemokine ligand 12, which is also known as stromal cell-derived factor 1 (SDF-1). The CXC chemokine ligand 12/CXCR4 signaling pathway plays an important role in carcinogenesis, progression, and metastasis, which results in decreased overall survival (OS) and poor prognosis.¹³⁻¹⁵ Although the concomitant expression of CD133-CXCR4 and their prognostic role in prostate, pancreatic, colon, and renal cancers have been demonstrated,¹⁶⁻¹⁹ no related studies have been performed in ESCC. Our previous studies have proved that CXCR4 expression is significantly associated with prognosis in ESCC, as well as matrix metalloproteinase-9/vascular endothelial growth factor expression.¹⁵ In addition, we have also demonstrated that CXCR4 expression makes a significant difference in cancer migration and metastasis.¹³ Thus, on the basis of the previous achievements on CXCR4, we tried applying CD133 for further investigation of the existence of a population of CSCs in ESCC tissue and the cell line TE-1 by detecting the coexpression of CD133 and CXCR4. In addition, we attempted to determine the clinical relevance and prognostic value of concomitant high CD133-CXCR4 expression in ESCC.

MATERIALS AND METHODS

Tissue Samples and Follow-up

Primary ESCC tissue samples were obtained from 154 patients who underwent radical esophagectomy in the Department of Thoracic Surgery, the Zhongshan Hospital, Fudan University, Shanghai, China, in 2006. Those who received neoadjuvant therapy (1 patient received preoperative chemotherapy, and 3 patients received preoperative radiotherapy) or died (1 patient) in the perioperative period were excluded. All sections of paraffin-embedded primary tumor tissues were reviewed by 2 experienced pathologists with no knowledge of the patients' information or the purpose of the research. After surgery, patients were followed up every 3 months during the first year and every 6 months thereafter. Patient data included details related to sex, date of birth, tumor pathology, TNM stage (according to American Joint Committee on Cancer, 7th ed),²⁰ disease-free survival (DFS), defined as the time elapsed from the date of surgery to local relapse

or distant metastasis, and OS, defined as the time elapsed from the date of surgery to death. Complete information on patient survival was available for 138 patients, and 16 patients were lost to follow-up. The last date of follow-up was March 31, 2015. This study was approved by the ethics committee on human research of Zhongshan Hospital.

Immunohistochemical Staining and Evaluation

Immunohistochemical staining was performed as described by Lu and colleagues.¹³ CD133 antibody (Cloud-Clone Corp, Houston, Tex) was used to detect the expression of the related protein. The measurement standard used for positive staining intensity has been described.¹³ All cases were separated into a low expression level group (scores 0 and 1) and a high expression level group (scores 2 and 3) based on the measurement standard.

Cell Culture

The human ESCC cell line TE-1 was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin in a humidified incubator under 95% air and 5% CO₂ at 37°C.

Flow Cytometry and Fluorescence-Activated Cell Sorting

Cultured cells were digested with trypsin and centrifuged at 300g for 10 minutes. The cells were resuspended with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany), FcR blocking reagent was added to the resuspended cells, and the cells were incubated at 4°C for 15 minutes. Anti-CD133 antibody (Miltenyi Biotec) and anti-CXCR4 antibody (Miltenyi Biotec) were added to the cells, and the cells were incubated in the dark at 4°C for 30 minutes. After the cells were washed 3 times with MACS buffer, the cells were analyzed and sorted into 4 subgroups based on combined high/low CD133 expression and high/low CXCR4 expression by flow cytometry on a FACScan (BD Biosciences, Franklin Lakes, NJ).

Colony-Forming Assay

Approximately 1.5×10^2 cells from each subgroup were placed in a 60 × 15-mm cell culture dish (3 repeat wells for each subgroup). After incubation at 37°C for 14 days, the cells were washed 3 times with phosphate-buffered saline, fixed with methanol for 15 minutes, and stained with 0.1% crystal violet solution for 40 minutes. Colonies with more than 20 cells were counted. The relative colony formation ratio was calculated by the mean value of "the number of colonies in a random visual fields/corresponding original number of cultivated cells" from 5 random visual fields.

Transwell Assay

Transwell filters (Costar, Coring, NY) (6.5-mm insert, 8-μm polycarbonate membrane) were chosen for this experiment. Cells (5×10^4) were harvested in 200 μL of serum-free DMEM medium and placed in the upper chambers of the plates. Then, 600 mL of DMEM supplemented with 20% fetal bovine serum was added to the lower chambers of the plates. After the plates had been incubated at 37°C for 36 hours, cells that had migrated from the upper chamber into the pores of the membrane insert were fixed with paraformaldehyde and stained with crystal violet at room temperature for 30 minutes. The cells that invaded the Matrigel (BD Bioscience, Bedford, Mass) were counted in 5 randomly selected visual fields using an inverted microscope at 200× magnification. The experiments were performed independently 3 times.

Statistical Analysis

Statistical analysis was performed with the Stata 11.0 software program (StataCorp LP, College Station, Tex). Correlations among the

FIGURE 1. Immunohistochemical ($\times 200$) analysis of CD133 and CXCR4 expression in ESCC tissues from the 154 patients enrolled in the study. Typical images of each score are shown. A1-4, CD133 expression level from score 0 to 3. B1-4, CXCR4 expression level from score 0 to 3. *CD133*, Cluster of differentiation 133; *CXCR4*, C-X-C chemokine receptor type 4; *IHC*, immunohistochemical.

expression of CD133, CXCR4, or double-positive ($CD133^{+}CXCR4^{+}$) and clinical characteristics were assessed by the Wilcoxon (2 groups) or Kruskal-Wallis (> 2 groups) rank test. DFS and OS curves were drawn by the Kaplan-Meier product limit method. Univariate analysis was performed with the log-rank test. The effects of several risk factors (covariates), including age, sex, tumor size, tumor depth, lymph node metastasis, histologic grade, CD133 expression, CXCR4 expression, and postoperative chemotherapy on DFS and OS, were analyzed by Cox proportional-hazards regression. The data gathered in in vitro experiments were noted as mean $\pm$ standard deviation, and the Student *t* test was used to analyze the difference between each subgroup.

RESULTS

CD133 and CXCR4 Expression in Esophageal Squamous Cell Carcinoma Tissues and TE-1 Cell Line

In the 154 ESCC tissue samples, staining of CD133 was observed in the cytoplasm, whereas staining of CXCR4 was observed in the cytoplasm, nucleus, and membrane (Figure 1). High expression of CD133 was observed in 28.57% (44/154) of the samples, and high expression of CXCR4 was observed in 65.58% (101/154) of the samples.

FIGURE 2. CXCR4⁺ cancer cells exhibited high invasive potential, and CD133⁺CXCR4⁺ cancer cells exhibited high proliferative capacity. Fluorescence-activated cell sorting analysis data for CD133/CXCR4 expression for (A) double-negative staining and (B) double-positive staining. Numbers show the percentage of positive cells gated. C, D, Transwell assay shows the difference in invasive ability between subgroups. C, Representative figure from 3 independent experiments. D, Statistical analysis was performed as the mean number of cells in the lower chamber in 5 random visual fields (****P* < .001). E, F, Colony-forming assay demonstrated the difference in proliferation ability between subgroups. E, Representative figure from 3 independent experiments. F, Statistical analysis was performed as the mean value of “the number of colonies in a random visual fields/corresponding original number of cultivated cells” from 5 random visual fields. (****P* < .001). *mIgG*, Membrane IgG; *CXCR4*, C-X-C chemokine receptor type 4; *CD133*, cluster of differentiation 133; *NS*, not significant.

TABLE 1. Analysis of associations between CD133/CXCR4 expressions and clinicopathologic features

Clinicopathologic features		CD133 expression		<i>P</i> value	CXCR4 expression		<i>P</i> value
		Low (%)	High (%)		Low (%)	High (%)	
Total	154	110 (71)	44 (29)		53 (34)	101 (66)	
Age							
≤60 y	76	56 (36)	20 (13)	.542	29 (19)	47 (31)	.336
>60 y	78	54 (35)	24 (16)		24 (16)	54 (35)	
Sex							
Male	119	81 (53)	38 (25)	.090	36 (23)	83 (54)	.046
Female	35	29 (19)	6 (4)		17 (11)	18 (12)	
Tumor size							
<3 cm	69	53 (34)	16 (10)	.184	29 (19)	40 (26)	.074
≥3 cm	85	57 (37)	28 (18)		24 (16)	61 (40)	
Tumor depth							
T1	12	10 (7)	2 (1)	.902	9 (6)	3 (2)	.003
T2	41	29 (19)	12 (8)		18 (12)	23 (15)	
T3	95	67 (44)	28 (18)		25 (16)	70 (46)	
T4	6	4 (3)	2 (1)		1 (1)	5 (3)	
Lymph node metastasis							
No	91	67 (44)	24 (16)	.470	43 (28)	48 (31)	.000
Yes	63	43 (28)	20 (13)		10 (7)	53 (34)	
TNM stage							
IA + IB	12	10 (7)	2 (1)	.403	8 (5)	4 (3)	.000
IIA + IIB	83	61 (40)	22 (14)		36 (23)	47 (31)	
IIIA + IIIB + IIIC	59	39 (25)	20 (13)		9 (6)	50 (32)	
Histologic grade							
I	14	10 (7)	4 (3)	.977	7 (5)	7 (5)	.426
II	114	82 (53)	32 (21)		38 (25)	76 (49)	
III	26	18 (12)	8 (5)		8 (5)	18 (12)	

CD133, Cluster of differentiation 133; CXCR4, C-X-C chemokine receptor type 4.

Coexpression of high levels of CD133 and CXCR4 was observed in 20.78% (32/154) of the samples.

To analyze the expression of CD133 and CXCR4 in vitro, we picked up TE-1 cell lines for inspection. In the analysis by flow cytometry, TE-1 cells were separated into the following 4 subgroups: CD133⁺CXCR4⁺ (1.20%), CD133⁺CXCR4⁺ (7.72%), CD133⁺CXCR4⁺ (0.40%), and CD133⁺CXCR4⁺ (90.68%) cancer cells (Figure 2, A and B). The low proportion of the cells with coexpression of CD133 and CXCR4 was in line with the characteristics of CSCs. Then, we isolated each subgroup through fluorescence-activated cell sorting.

CXCR4⁺ Cancer Cells Show High Invasive Potential and CD133⁺CXCR4⁺ Cancer Cells Show High Proliferative Capacity

To investigate the invasive potential and proliferative capacity among different subgroups, Transwell and colony-forming assays were performed. As shown in Figure 2, C and D for the Transwell assay, the numbers of migrated cells in the CXCR4⁺ subgroups (CD133⁺CXCR4⁺ and CD133⁺CXCR4⁺) were significantly larger than those in the CXCR4⁺ subgroups (CD133⁺CXCR4⁺ and CD133⁺CXCR4⁺; *P* < .001).

No significant difference was found between the CD133⁺CXCR4⁺ and CD133⁺CXCR4⁺ subgroups or between the CD133⁺CXCR4⁺ and CD133⁺CXCR4⁺ subgroups. As shown in Figure 2, E and F for the colony-forming assay, the numbers of visible colonies for the CD133⁺CXCR4⁺ cells outnumbered the other subgroups significantly (*P* < .001). No significant difference was found among the CD133⁺CXCR4⁺, CD133⁺CXCR4⁺, and CD133⁺CXCR4⁺ subgroups. These results suggested that the cells that expressed CXCR4 exhibited high invasive potential, and that the cells that coexpressed CD133 and CXCR4 exhibited high proliferative capacity.

High Coexpression of CD133 and CXCR4 Predicts Poor Prognosis of Patients With Esophageal Squamous Cell Carcinoma

During the follow-up, 99 patients had recurrence or metastasis. The location of metastasis included the supraclavicular lymph node, mediastinal lymph node, liver, lung, skeleton, and brain. In addition, 95 patients died of ESCC. On analyzing the relationship between clinicopathologic features and CD133/CXCR4 expression, we found that high CXCR4 expression had a significant

TABLE 2. Analysis of the associations between concomitant CD133-CXCR4 expression and tumor-associated factors

Clinicopathologic features		Concomitant CD133-CXCR4 expression		P value
		Low (%)	High (%)	
Total	73	41 (27)	32 (21)	
Tumor size				
<3 cm	37 (51)	25 (16)	12 (8)	.048
≥3 cm	36 (49)	16 (10)	20 (13)	
Tumor depth				
T1	9 (12)	8 (5)	1 (1)	.156
T2	24 (33)	15 (10)	9 (6)	
T3	39 (53)	18 (12)	21 (14)	
T4	1 (0)	0 (0)	1 (1)	
Lymph node metastasis				
No	51 (70)	35 (23)	16 (10)	.001
Yes	22 (30)	6 (4)	16 (10)	
TNM stage				
IA + IB	10 (14)	8 (5)	2 (1)	.002
IIA + IIB	42 (57)	28 (18)	14 (9)	
IIIA + IIIB + IIIC	21 (29)	5 (3)	16 (10)	
Histologic grade				
I	11 (15)	7 (5)	4 (3)	.818
II	52 (71)	29 (19)	23 (15)	
III	10 (14)	5 (3)	5 (3)	

CD133, Cluster of differentiation 133; CXCR4, C-X-C chemokine receptor type 4; TNM, tumor-node-metastasis.

association with tumor depth ($P < .01$), lymph node metastasis ($P < .01$), and TNM stage ($P < .01$), but CD133 expression did not (Table 1). To further analyze the effect of concomitant CD133-CXCR4 expression on the tumor-associated factors, we separated the patients into the CD133^{high}/CXCR4^{high} and CD133^{low}/CXCR4^{low} groups. The results revealed that concomitant high CD133-CXCR4 expression was significantly correlated with tumor size ($P < .05$), lymph node metastasis ($P < .01$), and TNM stage ($P < .01$) (Table 2).

In univariate analysis, we found that the DFS and OS in the CD133^{high}/CXCR4^{high} and CXCR4^{high} groups were significantly lower than those in the CD133^{low}/CXCR4^{low} and CXCR4^{low} groups ($P < .01$), whereas no significant difference was found between the CD133^{high} and CD133^{low} groups. However, the Kaplan–Meier curves demonstrated that patients with high CD133 expression had a relatively lower 5-year DFS/OS and median DFS/OS than the patients with low CD133 expression (DFS: 36.6% vs 40.5%, 28 vs 29 months; OS: 38.6% vs 43.7%, 32 vs 41 months). Although the 5-year DFS and OS of the patients with concomitant high CD133-CXCR4 expression were not lower than those of the patients with single high CXCR4 expression (DFS: 31.8% vs 27.7%; OS: 30.5% vs 30.1%), the median DFS and OS of the patients with concomitant high CD133-CXCR4 expression were shorter than those of the patients with single high CXCR4 expression (DFS: 19 vs 22 months; OS: 24 vs 26 months),

which indicated that CD133 might contribute to the poor prognosis of the patients (Figure 3, Table 3). Tumor depth, lymph node metastasis, and TNM stage were negatively correlated with patients' DFS and OS ($P < .01$) (Table 3). We applied multivariate analysis and found that lymph node metastasis was an independent factor for predicting prognosis (Table 4).

DISCUSSION

CSCs, also known as tumor-initiating cells, are defined as a rare subpopulation of cells characteristic of stem-like behavior. CSCs are considered to be a source of recurrence and metastasis in the case of malignant tumors. CD133 and CXCR4 have been widely used together as markers for CSCs in some malignant tumors, such as prostate cancer, pancreatic cancer, renal cancer, and colon cancer.¹⁶⁻¹⁹ In this study, immunohistochemical and flow cytometry results demonstrated that CD133 and CXCR4 were both expressed in patient samples and the ESCC cell line TE-1. The Transwell assay showed that the cells expressing CXCR4 had high invasive potential, and the colony-forming assay showed that the cells coexpressing CD133 and CXCR4 had high proliferative capacity. Through univariate and multivariate Cox analyses, we proved that concomitant high CD133-CXCR4 expression in ESCC tissue correlated with poor prognosis. These results indicated that CD133⁺CXCR4⁺ ESCC cells might have a high potential for migration and proliferation and, consequently, may predict poor prognosis of patients with ESCC.

To combine the basic experimental findings with clinical phenomena, we found that colony-forming assay results demonstrated that CD133⁺CXCR4⁺ cancer cells showed a higher proliferative capacity than the other subgroups. These basic experiment results corresponded to the size of the tumor. As shown in Tables 1 and 2, tumor size was associated with concomitant CD133-CXCR4 expression, but single CD133 expression or CXCR4 expression made no significant difference on tumor size, which was parallel to our findings in vitro. The Transwell assay demonstrated that CXCR4⁺ cancer cells showed a higher invasive ability than CXCR4⁻ cells, corresponding to the lymph node metastasis. Clinically, lymph node metastasis was associated with single CXCR4 expression and concomitant CD133-CXCR4 expression, but single CD133 expression made no significant difference on lymph node metastasis, which was also parallel to our findings in vitro.

CD133, a 5-transmembrane glycoprotein, is often expressed on hematopoietic stem cells and endothelial stem cells. Accumulated studies have reported CD133 as a marker for CSCs in various cancers.⁴⁻¹² Our data showed that CD133⁺CXCR4⁺ cancer cells had a higher proliferative capacity than the other subgroups in ESCC, which is parallel to the characteristics of CSCs.

FIGURE 3. DFS and OS curves of 154 enrolled patients with ESCC, obtained by Kaplan–Meier product limit method, were compared according to CD133 expression (A, B), CXCR4 expression (C, D), and concomitant CD133–CXCR4 expression (E, F). Red and blue dotted lines delineate the confidence limits. Black dotted lines mark the median survival. CD133, Cluster of differentiation 133; CXCR4, C-X-C chemokine receptor type 4.

Moreover, the receptor for SDF-1, CXCR4, plays an important role in the migration and metastasis in several cancers. For example, CXCR4 expression correlates with intrahepatic cholangiocarcinoma progression and

metastasis via the SDF-1/CXCR4 signaling pathway.²¹ A similar role of CXCR4 also has been detected in breast and thyroid.^{22,23} In our previous research, we proved that CXCR4⁺ cells showed a significant strong migration

TABLE 3. Univariate analysis of patients' survival

Variables	No. of patients	DFS			OS		
		Survival (5 y, %)	95% CI	P value	Survival (5 y, %)	95% CI	P value
Age							
≤60 y	76	46.0	34.2-56.9	.067	48.8	36.7-59.8	.020
>60 y	78	33.2	22.9-43.9		35.8	25.2-46.6	
Sex							
Male	119	50.1	32.4-65.5	.100	55.5	37.2-70.4	.062
Female	35	36.2	27.4-45.0		38.3	29.3-47.2	
Tumor size							
<3 cm	69	38.0	26.3-49.7	.433	42.2	30.0-53.8	.272
≥3 cm	85	40.4	29.8-50.7		42.3	31.5-52.7	
Tumor depth							
T1	12	73.3	37.9-90.6	.002	90.9	50.8-98.7	.000
T2	41	51.2	35.2-65.2		56.1	39.7-69.6	
T3	95	31.2	21.7-41.0		30.8	21.3-40.8	
T4	6	16.7	0.8-51.7		16.7	0.8-51.7	
Lymph node metastasis							
No	91	58.3	47.3-67.8	.000	59.0	47.8-68.5	.000
Yes	63	11.6	4.9-21.5		18.0	9.5-28.9	
TNM stage				.000			.000
IA + IB	12	90.9	50.8-98.7		90.9	50.8-98.7	
IIA + IIB	83	50.3	38.8-60.7		56.3	44.7-66.4	
IIIA + IIIB + IIIC	59	12.9	5.6-23.5		11.9	4.9-22.2	
Histologic grade							
I	14	69.3	37.0-87.3	.071	76.6	43.3-91.9	.048
II	114	36.1	27.2-45.1		38.3	29.2-47.4	
III	26	37.8	19.7-55.8		41.4	22.5-59.4	
CD133 expression							
Low	110	40.5	31.1-49.7	.521	43.7	34.1-53.0	.489
High	44	36.6	22.3-51.0		38.6	24.0-53.0	
CXCR4 expression							
Low	53	61.1	46.3-72.9	.000	64.7	49.9-76.2	.000
High	101	27.7	19.1-37.0		30.1	21.1-39.5	
CD133 and CXCR4 expression							
Both low	41	64.5	47.4-77.3	.006	66.5	49.3-79.1	.003
Both high	32	31.8	16.1-48.8		30.5	15.1-47.6	
Postoperative chemotherapy							
No	87	44.6	33.7-54.9	.092	48.1	37.0-58.3	.109
Yes	67	32.7	21.6-44.3		34.5	23.0-46.2	

DFS, Disease-free survival; OS, overall survival; CI, confidence interval; TNM, tumor-node-metastasis; CD133, cluster of differentiation 133; CXCR4, C-X-C chemokine receptor type 4.

potential in the ESCC cell line EC109 but had no effect on proliferation.¹³ In this study, we applied CD133 for further investigation and found that CD133⁺CXCR4⁺ and CD133⁻CXCR4⁺ cancer cells possessed high invasive potential, in accordance with the previous results. We achieved further findings that CD133⁺CXCR4⁺ cells possess higher proliferative capacity than the other 3 subgroups (CD133⁻CXCR4⁺, CD133⁺CXCR4⁻, and CD133⁻CXCR4⁻), which made a step forward on the basis of previous studies.¹³

More researchers are investigating the concomitant CD133-CXCR4 expression in some cancers and have detected the CSCs, characteristics of the double-positive (CD133⁺CXCR4⁺) cancer cells. Miki and colleagues¹⁶

found that in human prostate cancers, CD133⁺ cells were characteristic of CSCs. CXCR4 expression was detected in CD133⁺ cells, and it was found that CD133⁺CXCR4⁺ cells were prone to induce migration in vitro. Hermann and colleagues¹⁷ identified CD133⁺CXCR4⁺ cancer cells and determined the metastatic phenotype of the pancreatic cancer. Zhang and colleagues¹⁸ demonstrated that the stemness properties, including clonogenic and tumorigenic capacities, were only related to CD133⁺ cells in a colon cell line, independently of CXCR4 expression. However, Zheng and colleagues²⁴ concluded that CXCR4⁺ cells were up to the standard of CSC properties alone in glioma, including colony formation, sphere generation, and xenograft tumor generation, irrespective of CD133 expression. Thus, the

TABLE 4. Multivariate analysis of patients' survival

Variables	DFS			OS		
	HR	95% CI	P	HR	95% CI	P
Age	1.490	0.933-2.378	.095	1.788	1.099-2.911	.019
Sex	1.012	0.596-1.716	.966	1.066	0.622-1.827	.816
Tumor size	0.861	0.567-1.308	.483	0.851	0.555-1.306	.461
Tumor depth	1.332	0.947-1.873	.100	1.661	1.151-2.396	.007
Lymph node metastasis	2.570	1.649-4.006	.000	2.134	1.368-3.329	.001
Histologic grade	1.291	0.864-1.930	.213	1.341	0.877-2.052	.175
CD133 expression	1.107	0.711-1.724	.651	1.076	0.682-1.697	.753
CXCR4 expression	1.421	0.875-2.308	.156	1.432	0.867-2.363	.161
Postoperative chemotherapy	1.311	0.824-2.085	.253	1.366	0.849-2.198	.199

DFS, Disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CD133, cluster of differentiation 133; CXCR4, C-X-C chemokine receptor type 4.

biological roles of the coexpression of CD133 and CXCR4 cells are not completely consistent in different cancer cells. Our results demonstrated that the properties of CD133⁺CXCR4⁺ cancer cells are in accordance with the properties of CSCs. Therefore, a therapy that targets the subgroups coexpressing CD133 and CXCR4 might be an effective way to inhibit the proliferation and migration of cancer cells.

Zhang and colleagues¹⁸ further proved that the epithelial-mesenchymal transition contributed to the invasive capacity of CD133⁺CXCR4⁺ cells and that the metastatic behavior could be inhibited by AMD3100, a CXCR4 antagonist, which indicated the important role of the SDF-1/CXCR4 signaling pathway in facilitating the migration potential. However, it is unclear how these pathways interact and whether there are other mechanisms that have not been revealed. Therefore, more in-depth analysis is required to comprehensively understand the function of CSCs in tumor progression.

The prognostic role of coexpression of CD133 and CXCR4 also has been revealed in some cancers. In colon cancers, Zhang and colleagues¹⁸ found that the proportion of CD133⁺CXCR4⁺ was negatively correlated with 2-year survival for patients. In this study, we excluded patients who received neoadjuvant therapy, which might interfere with the original expression of CD133/CXCR4 and be against our original aim to find the relationship between original CD133/CXCR4 expression with the clinical features and prognosis. Our follow-up data demonstrated the similar results that the 5-year OS and DFS in the CD133^{high}/CXCR4^{high} group were significantly lower than those in the CD133^{low}/CXCR4^{low} group. In addition, in the multivariate Cox regression analysis, we found that lymph node metastasis was an independent prognostic factor in patients with ESCC. Although no significant association was found between CD133 expression and prognosis according to the univariate

analysis, the Kaplan–Meier curves demonstrated that patients with high CD133 expression had relatively lower 5-year DFS/OS and median DFS/OS than patients with low CD133 expression (DFS: 36.6% vs 40.5%, 28 vs 29 months; OS: 38.6% vs 43.7%, 32 vs 41 months). Although the 5-year DFS and OS of the patients with concomitant high CD133-CXCR4 expression were not lower than those of the patients with single high CXCR4 expression (DFS: 31.8% vs 27.7%; OS: 30.5% vs 30.1%), the median DFS and OS of the patients with concomitant high CD133-CXCR4 expression were shorter than those of the patients with single high CXCR4 expression (DFS: 19 vs 22 months; OS: 24 vs 26 months). Thus, we concluded that CD133 and CXCR4 may act together to facilitate the progression of ESCC and predict a poor prognosis in patients.

CONCLUSIONS

We have demonstrated that the cells coexpressing CD133 and CXCR4 possess the characteristics of CSCs. Meanwhile, the concomitant CD133-CXCR4 expression might be a novel marker for predicting a poor prognosis in patients with ESCC, and CD133 and CXCR4 may serve as potential therapeutic targets.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: cancer stem-like cells, CD133, CXCR4, esophageal squamous cell carcinoma

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