Monocarboxylate transporter 1 is an independent prognostic factor in esophageal squamous cell carcinoma

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Abstract. The monocarboxylate transporter 1 (MCT1) has been reported to have significant prognostic value in several solid tumors. The present study aimed to explore its clinical significance in esophageal squamous cell carcinoma (ESCC). After acquiring and analyzing *MCT1* (solute carrier family 16 member; *SLC16A1*) mRNA expression in The Cancer Genome Atlas (TCGA) database, the prognostic potential of MCT1 was assessed by immunohistochemistry (IHC). The impact of the knockdown of MCT1 by shRNA was evaluated using Cell Counting Kit-8 (CCK-8) and colony formation assays to determine whether MCT1 suppression affected the proliferation and survival of ESCC cells. MCT1 expression was found to correlate with T stage (P=0.005), N stage (P=0.036) and TNM stage (P=0.035). Kaplan-Meier survival analysis showed that patients in a high-MCT1 group had a lower overall survival (OS) (P<0.001) and lower progression-free survival (PFS) (P<0.001). The results of univariate and multivariate Cox regression analyses demonstrated that MCT1 is an independent prognostic factor for OS (P=0.001 and 0.01) and PFS (P=0.001 and 0.012). Downregulation of MCT1 suppressed proliferation and survival of ESCC cells *in vitro*. The proliferation rate and colony numbers were decreased in the sh-MCT1 groups (all P<0.05). Downregulation of MCT1 suppressed VEGF expression (all P<0.05). MCT1 may act as a biomarker for ESCC to identify patients with poor outcomes.

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Introduction

Esophageal cancer is a dangerous disease, based on both morbidity and mortality. Worldwide, esophageal cancer is currently the eighth most prevalent cancer and the sixth leading cause of cancer-related deaths (1). The latest epidemiological survey of malignant tumors showed that the incidence of esophageal cancer in China is 477,900 (out of 1,37 billion), with 375,000 deaths/year (2). It is estimated that the number of new cases of esophageal cancer will reach 2,110,000 by 2025 worldwide. Although the incidence of many other types of cancer will decrease during this time, the prevalence of esophageal cancer is expected to increase by 140% (3). This cancer includes two pathological types: Squamous cell carcinoma (ESCC) and adenocarcinoma (EAC). The incidence of the latter has significantly increased over the last 40 years; however, squamous cell carcinoma (ESCC) remains the principal type of esophageal cancer (1). In the highest-prevalence areas for esophageal cancer, often called the 'esophageal cancer belt' (including China) 90% of these tumors are ESCC (4). Endoscopic management and new diagnostic imaging technologies may aid the early diagnosis and improve the overall survival. Nevertheless, the 5-year survival rate remains only 18% (5). Because of the atypical early symptoms of esophageal cancer and the absence of specific tumor markers for early diagnosis, most patients are diagnosed at advanced stages, resulting in poor prognosis (6). There are no reliable biomarkers for the early clinical diagnosis of esophageal cancer; therefore, this area requires much work.

Deregulating cellular energetics is an important hallmark of cancer. This concept can be thought of as equivalent to the concept of sustaining proliferative signaling, activating invasion and metastasis, angiogenesis and evasion of apoptosis. Through a study by Hanahan and Weinberg, it was reported that most cancer cells are powered by aerobic glycolysis (the so-called Warburg effect) (7,8). Cancer cells rely on glycolysis to provide energy, producing a large amount of lactate. When lactate is transported into the extracellular milieu to avoid intracellular acidification and apoptosis, an

acidic microenvironment is formed. Monocarboxylate transporters 1-4 (MCT1-4) are involved in this process (9).

MCTs belong to the SLC16 family of genes, including at least 14 members. MCT1-MCT4 are thought to be proton transporters that regulate the transmembrane transport of lactate, pyruvate and ketone bodies (9). The function of MCTs in maintaining the homeostasis of cells in normal tissues has been studied in detail, but there is little insight regarding their role in cancer tissues. In acidic tumor microenvironments, MCTs not only play roles in maintaining the hyper-glycolytic acid-resistant phenotype of cancer cells, but also play an important role in maintaining high glycolytic rates that depend on mediating lactate efflux (9). MCT1 (solute carrier family 16 member; SLC16A1) and MCT4 (SLC16A4) mediate the extrusion of large amounts of lactic acid from malignant tumor cells, forming an acidic tumor microenvironment, thereby increasing the invasiveness and mobility of malignant cells (10).

Deregulating cellular energetics of cancer cells is an emerging field of research, and research regarding the expression and functional role of MCT1-4 recently have been conducted. Several studies of the mechanisms of their effects have been reported for several malignancies, including non-small cell lung cancer (NSCLC), bladder cancer, osteosarcoma and breast cancer (11-14). Based on these studies, we believe that MCT1-4 may play a crucial role in tumor biological behavior and be a potential target for cancer diagnosis and therapeutics. The objective of the present study was to evaluate the expression of MCT1 in ESCC as well as its prognostic implications.

Materials and methods

Ethics statement. The research protocol of the present study was approved by the Ethics Committee of Qilu Hospital, and all patients signed written informed consents before enrollment. Patient information was anonymized and unidentifiable prior to analysis.

Patients and tissue samples. The primary tumor tissues and the corresponding para-carcinoma tissues were obtained from 103 patients, who had confirmed diagnoses of ESCC at the Qilu Hospital of Shandong University from February 2010 to December 2011 and had not received any neoadjuvant-therapy. All samples were verified by pathological assessment. All 103 patients were followed up for at least five years. Other information was collected from clinical and pathological records. Staging of the tumors was according to the American Joint Committee on Cancer (AJCC, 7th edition).

Cell lines and culture. Human esophageal squamous cell carcinoma (ESCC) cell strains KYSE-150 and Eca-109 were purchased from the China Center for Type Culture Collection (Beijing, China) in 2017. All cells were verified by short tandem repeat analysis. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were cultivated in a 37°C humid incubator containing 5% CO_2.

Cell transfection. Eca109 and KYSE-150 cell lines were transfected with plasmids purchased from GeneCopoeia, with shRNA targeting the MCT1 gene and shRNA as control using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection procedure was carried out in accordance with the manufacturer's instructions. After transfection, the cells were cultured and were harvested within 48-72 h for subsequent analysis.

Cell Counting Kit-8 (CCK-8) assay. According to the instructions of the CCK-8 kit (BestBio, Shanghai, China), the cells in logarithmic growth phase were digested to construct a single-cell suspension and the cells were counted, after which they were seeded at a density of 1,000 cells/well on 96-well plates. In each 96-well, 100 μl of the suspension was placed. Subsequently, the multiplication capacity of Eca109 and KYSE-150 cells was assessed at 24, 48, 72, 96 and 120 h. We added 10 μl CCK-8 solution into 100 μl fresh medium to each well, followed by additional incubation for 2 h, and then measured the absorbance at 450 nm with a Thermo Scientific Varioskan Flash spectrophotometer (Thermo Scientific, Inc., Vantaa, Finland).

Colony formation assay. Cells that were digested by proteases were made into a single-cell suspension, and 500 cells were seeded per well of a 6-well plate. Cells were fixed with methanol, stained with crystal violet, and counted after culture for 2 weeks. We then counted the number of colonies containing >50 cells, defined as a clone (ImageJ 1.47v software; NIH; National Institutes of Health, Bethesda, MD, USA).

Western blotting. Cells were lysed with cold RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and phenylmethylsulfonyl fluoride (PMSF) (dilution 1:100) for 30 min on ice after washing three times with PBS. Cell debris was discarded after centrifugation (12,000 x g, for 15 min) at 4°C. The supernatant was stored in a new EP tube. Protein concentrations in the supernatant were determined with the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Boiled mixtures of protein and sample buffer were electrophoresed on 10% SDS-PAGE gels at 80 V for 120 min. The proteins were transferred to PVDF membranes on ice for 2 h and blocked with 5% dried skimmed milk at room temperature for 1.5 h. The membranes were washed with TBST (pH 7.4) three times and were incubated with primary antibodies at 4°C overnight. Secondary antibodies [peroxidase-conjugated goat anti-rabbit IgG (H+L); dilution 1:5,000; cat. no. ZB-2301; peroxidase-conjugated goat anti-mouse IgG (H+L); dilution 1:5,000; cat. no. ZB-2305; both from ZSBIO, Beijing, China] were added to the membranes and incubated at room temperature for 1 h. Subsequently, the membranes were exposed with an enhanced chemiluminescence reaction (ECL) kit, and the gray-levels of the protein bands were analyzed using ImageJ 1.47 v software (NIH; National Institutes of Health, Bethesda, MD, USA). The primary antibodies included: Rabbit anti-MCT1 polyclonal antibody (dilution 1:1,000; cat. no. 20139-1-AP; ProteinTech Group Inc., Chicago, IL, USA), rabbit anti-ACTB polyclonal antibody (dilution 1:1,000; cat. no. ab8227; Abcam, Cambridge, UK) and mouse anti-VEGF monoclonal antibody

(dilution 1:500; cat. no. sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA extraction and qRT-PCR. Total cellular RNA in KYSE-150 and Eca109 cell lines was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). One microgram of RNA was reverse-transcribed by SYBR-Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan). All operations were carried out on a Bio-Rad Single Color Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The forward primer: 5'-CACCACCAGCGAAGT GTCAT-3' was used to prepare the first strand synthesis. The reverse primer: 5'-ATCAAGCCACAGCCTGACAA-3' was applied for DNA amplification.

Immunohistochemistry (IHC). Samples were stained for immunohistochemistry for MCT1. All applied antibodies were validated in paraffin-embedded tissue for immunohistochemistry (IHC) by the manufacturer. Paraffin-embedded slides were dewaxed with xylene and rehydrated with graded ethanol, and then antigen retrieval was conducted using the microwave heating technique. Sections were then incubated with a rabbit anti-MCT1 polyclonal antibody (dilution 1:100; cat. no. 20139-1-AP; ProteinTech Group Inc.) overnight at 4˚C. Subsequently, after swilling with PBS three times, we added the secondary antibody [peroxidase-conjugated goat anti-rabbit IgG (H+L); dilution 1:5,000; cat. no. ZB-2301; ZSBIO] to the slices and incubation was carried out at 37˚C for 30 min. The immunological reaction was visualized with diaminobenzidine (DAB) as a chromogenic agent and re-dyed with hematoxylin. The stain intensity of the stained slides was evaluated by two pathologists in a blinded manner, without prior knowledge of the basic data or clinical features of the patients. Five fields were selected at high magnification randomly, and the number of positive cells was counted and averaged. The staining intensity of the sections and the number of positive cells were both used to calculate the IHC score. The degree of staining was graded as: 0 (no staining), 1 (weak), 2 (moderate) and 3 (intense); the number of positive cells was classified as: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). The arithmetic product of these two scores was designated as the final score: Score 0-1 (-), score 2-4 (+), score 5-8 (++) and score 9-12 (+++). Samples with scores >8 (+++) were regarded as having overexpression.

Statistical analysis. All statistical analyses were conducted using SPSS 24.0 software (IBM Corp., Armonk, NY, USA). The bilateral χ^2 test was used to analyze the correlation of MCT1 expression and clinicopathological characteristics. The Kaplan-Meier method and log-rank analysis were used to draw the survival curves and explore the differences in overall survival (OS) and progression-free survival (PFS) between MCT1-overexpressing and MCT1-low-expressing groups. Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to ascertain the predictive value of MCT1. Univariable and multivariate Cox regression analyses were used to determine the significant factors and for calculating the hazard ratios (HRs). The various mRNA expression levels between sh-NC and sh-MCT1, the CCK-8 proliferation assay data and the colony formation

Table I. Correlation of MCT1 expression with the clinicopathological features of ESCC in the FFPE cancer tissues.

Clinicopathological features	MCT1 overexpression		P-value[a]
	No (scores ≤8) (n=42)	Yes (scores >8) (n=1)	
Sex			0.071
Female	10	25	
Male	32	36	
Age (years)			0.922
<60	19	27	
≥60	23	34	
History of drinking			0.054
No	16	35	
Yes	26	26	
History of smoking			0.703
No	17	27	
Yes	25	34	
T stage			**0.005[b]**
T1	4	7	
T2	25	16	
T3	10	23	
T4	3	15	
N stage			**0.036[b]**
N0	24	19	
N1	4	17	
N2	9	17	
N3	5	8	
TNM stage			**0.035[b]**
I	11	11	
II	16	16	
III	15	34	
Differentiation			0.527
Well	20	23	
Moderate	10	20	
Poor	12	18	

MCT1, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma; FFPE, formalin-fixed paraffin-embedded; TNM, Tumor, Node, Metastasis. [a]Chi-square test; [b]P<0.05. P-values in bold print indicate statistically significant correlations.

results were analyzed using a paired Student's t-test. P<0.05 was considered to indicate a statistically significant result.

Results

Association between MCT1 expression and clinicopathologic features. According to the TCGA database (http://ualcan.path. uab.edu/cgi-bin/TCGAExResultNew2.pl?genenam=SLC16A1 &ctype=ESCA), *MCT1* (*SLC16A1*) was upregulated in ESCC tissues compared to normal tissues (Fig. 1A; P<0.001). MCT1
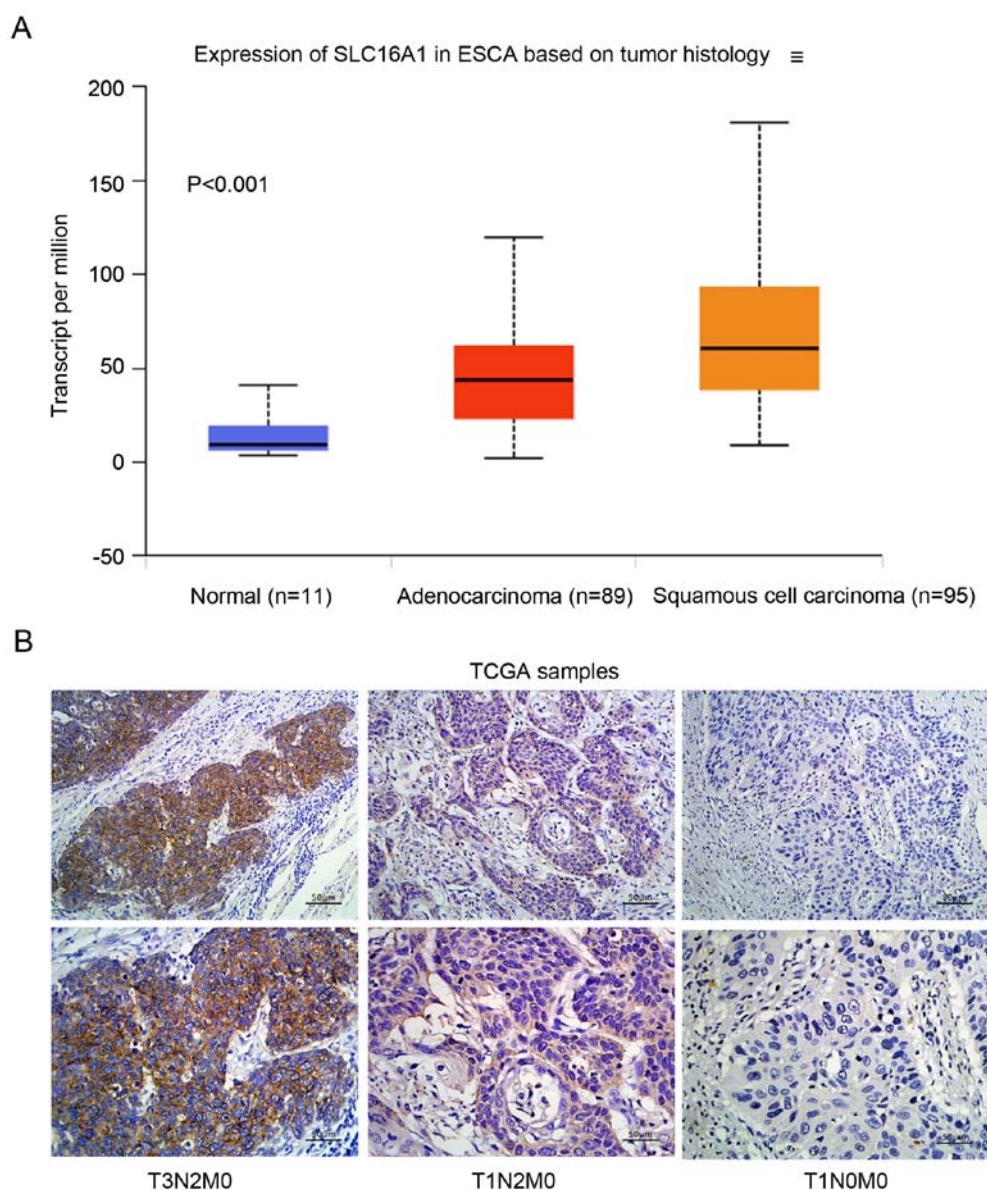
Figure 1. (A) *MCT1 (SLC16A1)* mRNA expression data in ESCC acquired from TCGA. (B) Representative immunohistochemistry of *MCT1* for ESCC tissues of different stages: The T1N0M0 and T2N1M0 ESCC tissues did not overexpress *MCT1*, while the T3N2M0 ESCC tissues exhibited overexpression of *MCT1*. *MCT1*, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma; TCGA, The Cancer Genome Atlas; ESCA, esophageal carcinoma; SLC16A1; solute carrier family 16 member.

was assessed on the cell membranes of ESCC tumor cells. Representative images of MCT1 immunohistochemistry are shown in Fig. 1B.

In total, the study enrolled 103 ESCC patients, 35 (33.98%) of whom were female, 68 (66.02%) were males, 46 (44.7%) were younger than 60 years, and 57 (55.3%) were older than 60 years. Among the 103 ESCC patients, 61 were in an MCT1 high-expression group (score >8) and 42 were in the low-expression group (score ≤8). In terms of survival, 37 (35.9%) patients were still alive at the end of the five-year follow-up period, while 66 (64.1%) patients died during this period. The survival time of all patients ranged from 6-80 months, and the median survival time was 40 months. The relationship between MCT1 expression and clinicopathologic features are shown in Table I. We found that there was a significant correlation between MCT1 expression and T stage (P=0.005), N stage (P=0.036) and TNM stage (P=0.035). Other clinicopathologic features

had no significant correlation with MCT1 expression, including sex, age, drinking and smoking history and tumor differentiation.

Prognostic value of MCT1. Overall survival (OS) and progression-free survival (PFS) impacted by MCT1 were determined by the Kaplan-Meier method and log-rank analysis (Fig. 2A and B). A lower 5-year OS was found in the MCT1 high-expression group (P<0.001). The same situation was observed for PFS (P<0.001). The ROC curve was drawn and the AUC values were used to determine the predictive efficiency of MCT1. AUC values for death and progression were 0.667 (P=0.005) and 0.648 (P=0.020), respectively (Fig. 2C and D). Sensitivity and specificity are documented in Fig. 2E.

The results of the univariate and multivariate Cox regression analyses are displayed in Table II. Upon univariate
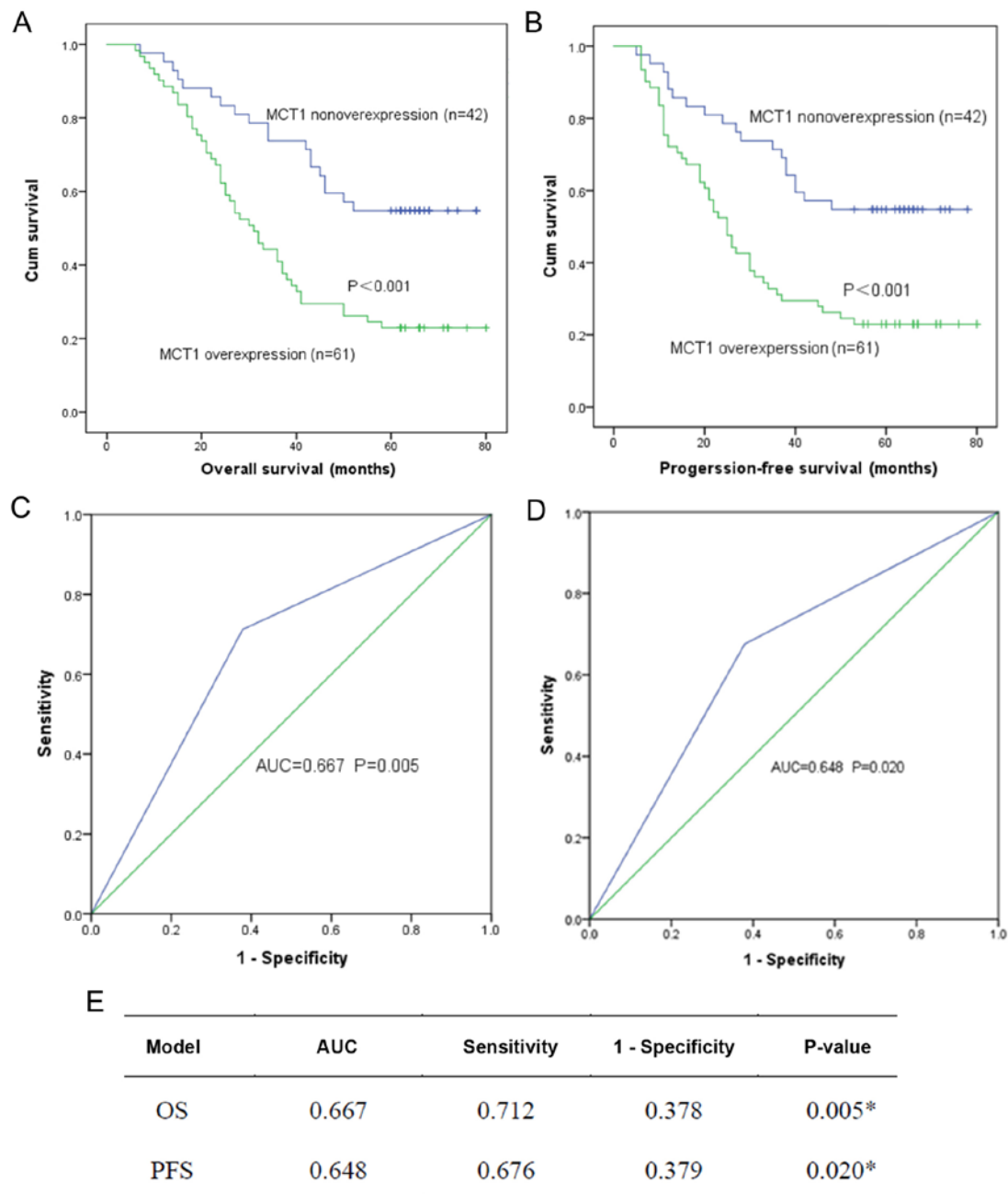
Figure 2. Kaplan-Meier curves and ROC curve analyses based on MCT1 expression in ESCC tissues. (A) Kaplan-Meier curve for OS. (B) Kaplan-Meier curve for PFS. (C) Receiver operating curve for OS. (D) Receiver operating curve for PFS. (E) Sensitivity and specificity for OS and PFS. AUC, area under the receiver operating characteristic (ROC) curve; MCTI, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma; OS, overall survival; PFS, progression-free survival.

analysis, the factors significantly related to OS were T stage (P<0.001), N stage (P<0.001) and MCT1 expression (P=0.001). T stage (P<0.001), N stage (P=0.003) and MCT1 expression (P=0.001) were verified to be significantly associated with PFS. Upon multivariate analysis, T stage was also found to be a significant prognostic marker for ESCC patients (OS: P=0.012, PFS: P=0.013). Multivariate analysis further confirmed that MCT1 may be a significant prognostic marker for ESCC patients in terms of OS (P=0.01) and PFS (P=0.012).

Downregulation of MCT1 inhibits the proliferation of ESCC Eca109 and KYSE-150 cells. To determine the transfection efficiency of the sh-MCT1 plasmids, the expression of MCT1 was verified by qRT-PCR (Fig. 3A) and western blotting (Fig. 3B and C) at the mRNA and protein levels, in KYSE-150 and Eca109 cells. The mRNA of *MCT1* was significantly lower following transfection of the sh-MCT1 plasmids in both cell lines (Eca109: 1.06±0.08 vs. 0.49±0.04, P<0.001; KYSE-150: 0.96±0.05 vs. 0.50±0.05, P<0.001, respectively). MCT1 protein expression was lower in the sh-MCT1 groups than levels in the sh-NC groups (MCT1/ACTB: Eca109: 0.72±0.08 vs. 0.43±0.04, P<0.001; KYSE-150: 0.7±0.08 vs. 0.41±0.04, P<0.001, respectively).

To explore the role of MCT1 in the proliferation of ESCC cells, we conducted a CCK-8 and colony formation

Table II. Univariate and multivariate analyses of prognostic variables based on clinical features and IHC scores of the ESCC cases.

Variable	OS Univariate analysis P-value	OS Multivariate analysis P-value	HR	95% CI	PFS Univariate analysis P-value	PFS Multivariate analysis P-value	HR	95% CI
Sex (female vs. male)	0.18	0.242	0.696	0.380-1.276	0.12	0.226	1.451	0.794-2.650
Age in years (<65 vs. ≥65)	0.84	0.59	1.167	0.665-2.050	0.952	0.629	0.871	0.499-1.522
Drinking (yes vs. no)	0.341	0.25	0.695	0.347-1.292	0.318	0.27	1.418	0.763-2.634
Smoking (yes vs. no)	0.845	0.345	1.346	0.726-2.495	0.827	0.353	0.745	0.401-1.386
T stage	**<0.001[a]**	**0.012[a]**			**<0.001[a]**	**0.013[a]**		
T1			1	Ref			1	Ref
T2		0.031[a]	4.281	1.143-16.037		0.032[a]	4.214	1.130-15.718
T3		0.006[a]	5.956	1.680-21.115		0.006[a]	5.858	1.659-20.692
T4		0.002[a]	8.023	2.196-29.309		0.002[a]	7.781	2.138-28.314
N stage	**<0.001[a]**	0.151			**0.003[a]**	0.179		
N0			1	Ref			1	Ref
N1		0.386	1.382	0.665-2.871		0.382	1.386	0.667-2.878
N2		0.025[a]	2.197	1.104-4.370		0.03[a]	2.138	1.077-4.247
N3		0.678	1.199	0.510-2.818		0.631	1.233	0.525-2.895
Differentiation	0.395	0.357			0.414	0.386		
Well			1	Ref			1	Ref
Moderate		0.96	1.016	0.536-1.926		0.93	1.029	0.546-1.939
Poor		0.217	0.635	0.308-1.307		0.243	0.651	0.317-1.338
MCT1 overexpression (scores >8)	**0.001[a]**	**0.01[a]**	2.273	1.213-4.258	**0.001[a]**	**0.012[a]**	0.45	0.240-0.841

IHC, immunohistochemistry; MCT1, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma; OS, overall survival; PFS, progression-free survival; HR, hazards ratio; CI, confidence interval. [a]$P<0.05$. P-values in bold print are indicative of significant correlations.

assays in KYSE-150 and Eca109 cell lines. The results of the CCK-8 assay showed that the OD values (Fig. 4A) of the sh-MCT1 groups were significantly lower than those of the sh-NC groups at 24, 48, 72, 96 and 120 h in both cell lines ($P<0.001$). Consistent with this finding, the number of clones in the Sh-MCT1 groups (Fig. 4B and C) were significantly lower (Eca109: 233±11 vs. 70±3, $P<0.0001$; KYSE-150: 249±15 vs. 72±4, $P<0.0001$, respectively). To find biochemical markers modified by MCT1 downregulation, we examined the expression of VEGF in the MCT1-silenced samples at the mRNA (Fig. 5A) and protein levels (Fig. 5B and C). The mRNA of VEGF was decreased significantly with sh-MCT1 plasmid transfection in both cell lines (Eca109: 1.193±0.07 vs. 0.69±0.03, $P<0.001$; KYSE-150: 1.28±0.04 vs. 0.68±0.03, $P<0.001$, respectively). The expression of VEGF protein was lower in the sh-MCT1 groups than that in the sh-NC groups (VEGF/β-tubulin: Eca109: 0.85±0.02 vs. 0.52±0.01, $P<0.001$; KYSE-150: 0.92±0.02 vs. 0.71±0.04, $P<0.001$, respectively). The results showed that the expression of VEGF was significantly decreased after MCT1 downregulation. In summary, the data suggested that MCT1 is a contributing factor to the proliferation of ESCC cells by modifying the expression of vascular endothelial growth factor (VEGF).

Discussion

MCT1 expression is a poor prognostic marker. We initially explored the expression of MCT1 (solute carrier family 16 member; SLC16A1) in esophageal cancer and its clinical significance. All 103 patients were followed up for at least five years to gather the follow-up data, and clinicopathologic data were obtained for survival analysis. This is the first study to carry out survival analysis on MCT1. We found that MCT1 was highly expressed in certain ESCC patients. Previous studies have shown that TNM stage plays an integral part in guiding stage-specific treatment protocols and has a major impact on overall survival (OS) (1). Tumor length and the number of lymph node metastases are indicative of a poor prognosis of esophageal cancer (15,16). In the present study, T staging (P=0.005), N staging (P=0.036) and TNM staging (P=0.035) were strongly correlated with the expression of MCT1. This suggests that MCT1 may be associated with unfavorable prognosis in esophageal cancer.
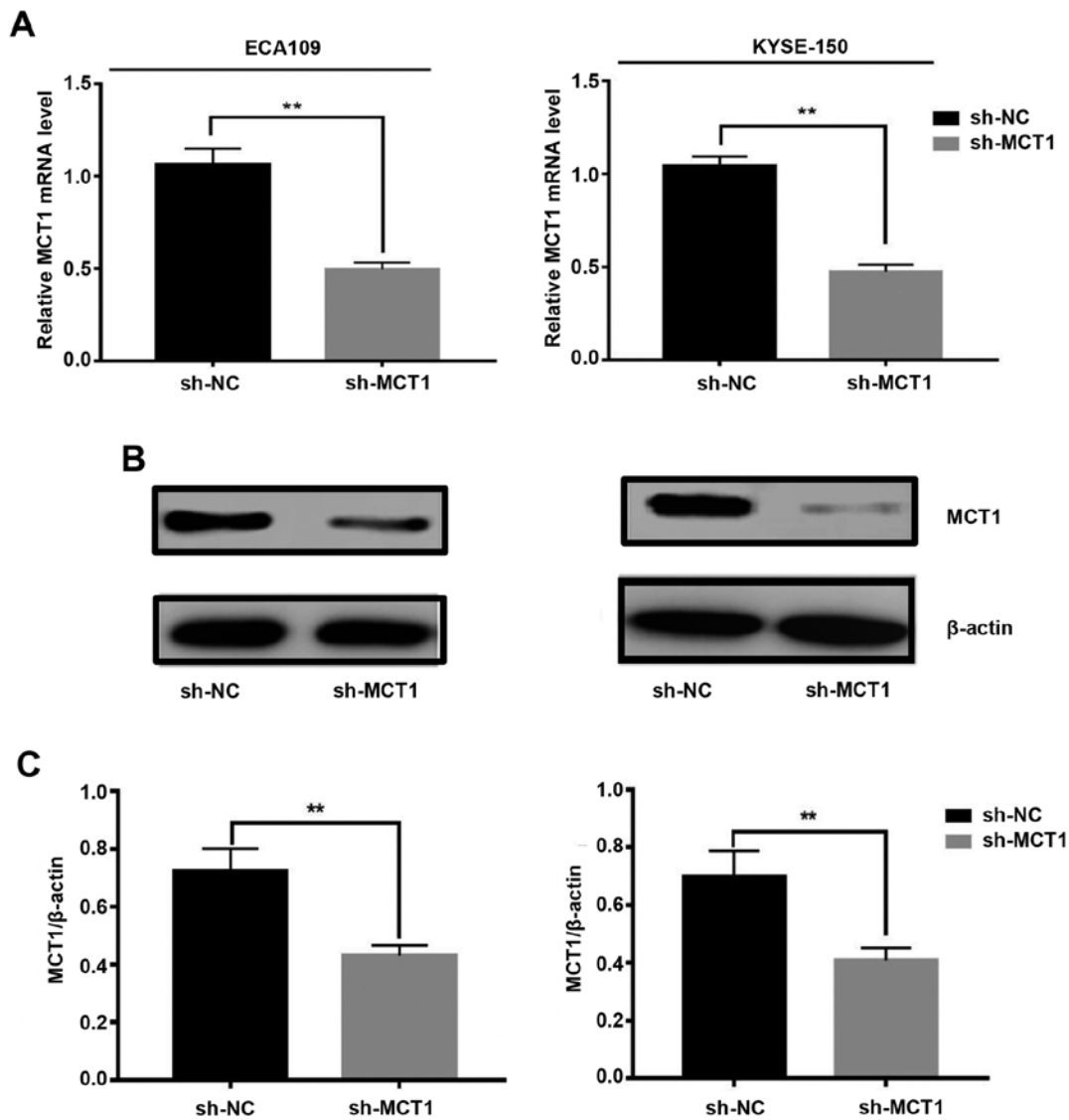
Figure 3. Downregulation effect of sh-MCT1 on ESCC Eca109 and KYSE-150 cell lines. (A) sh-MCT1 decreased mRNA expression of MCT1. (B and C) Expression of MCT1 protein in Eca109 and KYSE-150 cells was downregulated after sh-MCT1 transfection. **P<0.001. MCTI, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma.

High MCT1 expression is associated with poor outcomes. In previous studies, the expression and role of MCT1 have been adequately validated and explained in detail, especially for breast cancer, hepatocellular carcinoma, bladder cancer and glioblastoma; MCT1 was found to act as a biomarker of poor outcome, while in non-small cell lung cancer (NSCLC) it exhibited an opposite effect (11,12,14,17,18). By univariate analysis, we found that high expression of MCT1was associated with lower OS (P=0.001) and PFS (P=0.001). In addition, based on CCK-8 and colony formation assays, the expression of MCT1 and the proliferation of ESCC cells were positively correlated. This suggests that MCT1 may be useful as a prognostic biomarker, valuable for designing clinical trials using MCT1 inhibitors.

Possible mechanism underlying the unfavorable prognosis caused by MCT1. The present study is consistent with previous studies that revealed a strong association between MCT1 and unfavorable prognosis (19-21). High expression of MCT1 was accompanied by enhancement of proliferation, migration and invasiveness, revealing more aggressive tumor characteristics and worse prognosis based on the present and previous results (13,22-25).

Mechanistically, the function of MCT1 as a lactic acid transporter has been demonstrated to be closely related to tumor progression (14,17,21,26). Malignant cancers have been shown to exhibit characteristic alterations of metabolism, including the 'Warburg effect' and increased dependence on amino acid metabolism (7,27). First, the anaerobic glycolysis of tumor cells under normoxic conditions results in excess lactic acid in cells. In the present study, we found a high expression of MCT1 in ESCC, and there was observable MCT1 expression on the plasma membranes of KYSE-150 and Eca109 cells (Fig. 1B). As MCT1 is an ion transport-related molecule that releases protons to the extracellular medium, high expression of MCT1 may result in both a weakly alkaline intracellular pH (pHi) and an acidic extracellular pH (pHe). The function of tumor cells depends on the maintenance of an intracellular weak alkaline pH (23). Furthermore, previous studies have shown that
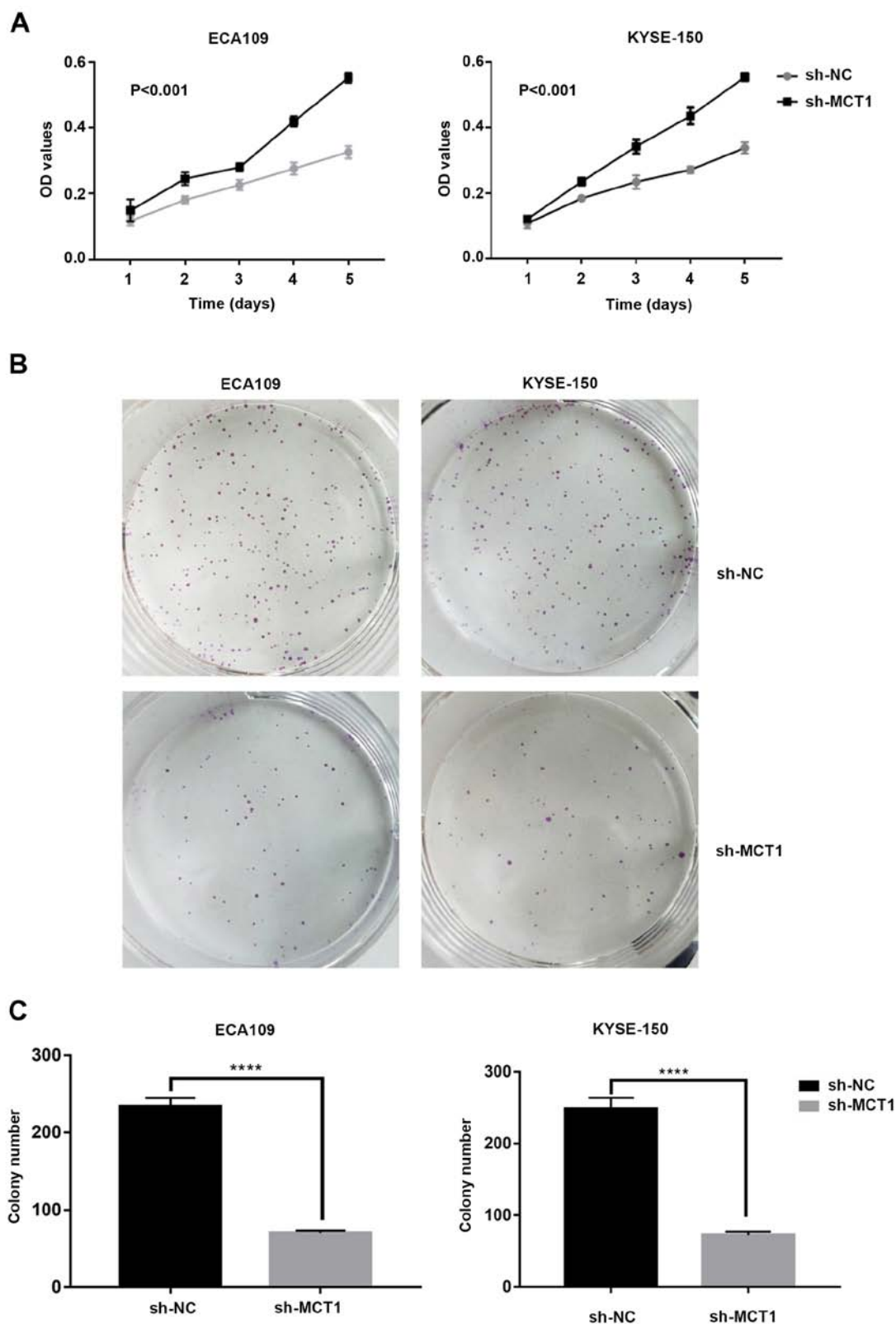
Figure 4. MCT1 regulates the proliferation ability of ESCC Eca109 and KYSE-150 cells *in vitro*. (A) The results of the CCK-8 assays in Eca109 and KYSE-150 cells. (B and C) Results of the colony formation assay in Eca109 and KYSE-150 cells. ****P<0.0001. MCTI, monocarboxylate transporter 1; ESCC, esophageal squamous cell carcinoma.

an acidic extracellular pH (pHe) increased the expression of vascular endothelial growth factor (VEGF), cathepsin B (CB), matrix metalloproteinase-2 and -9 (MMP-2 and -9), carbonic anhydrase 9 and interleukin-8 (IL-8), all of which have been found to be associated with enhanced tumor cell survival, migration and invasion (28-31). We found that downregulation of MCT1 resulted in decreased expression of VEGF (Fig. 5), which is an important factor in angiogenesis and tumor
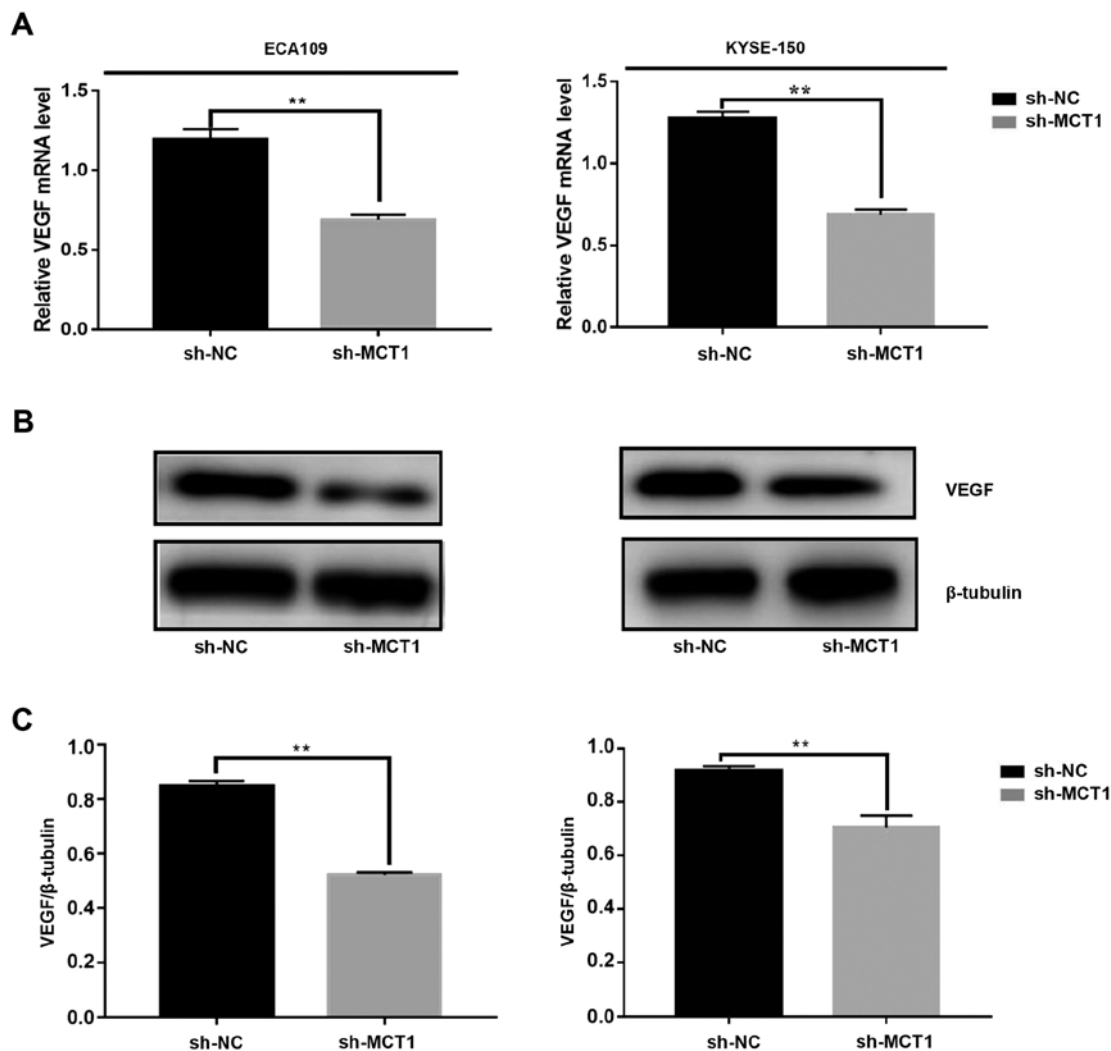
Figure 5. The inhibitory effect of MCT1 downregulation on VEGF. (A) sh-MCT1 decreased mRNA expression of VEGF in ESCC Eca109 and KYSE-150 cells. (B and C) Expression of VEGF protein in Eca109 and KYSE-150 cells was downregulated after sh-MCT1 transfection. **P<0.001. MCTI, monocarboxylate transporter 1; VEGF, vascular endothelial growth factor; ESCC, esophageal squamous cell carcinoma.

cell growth regulated by the VEGF-AKT-NF-κB signaling pathway (32). This may be a potential mechanism underlyling the suppression of ESCC cell proliferation caused by down-regulation of MCT1, but more convincing experiments need to be carried out.

Therefore, a large amount of the available data on the correlation between MCT1 and cancer are focused on the contribution of MCT1 as a transporter. Notably, apart from the primary transporting function, there is little evidence of transporters exhibiting tumor-promoting activities relying on other features. Nonetheless, a study conducted by Gray *et al* revealed a novel function of MCT1 independent of transporter activity. They explained that MCT1 regulated tumor migration by activating the HGF/c-Met pathways apart from its function as a proton transporter (33). The HGF/c-met pathway is believed to induce tumor cells to undergo epithelial-mesen-chymal transformation (EMT), characterized by absence of cell-cell adhesion, leading to more active tumor cell motility, invasion and metastasis (34). EMT is an important feature of radioresistance phenotypes in tumor cells and our finding that MCT1 activates the HGF/c-Met pathways may be a novel concept for future study.

Limitations and future directions. Normal esophageal tissues were not included in this study. All tissue specimens were paraffin-embedded esophageal cancer tissues preserved in the Pathology Department of Qilu Hospital, which were removed during esophagectomy. Whether the margin of the tumor resection was normal tissue was not verified, and the normal esophageal tissue that was determined could not be removed during surgery. Therefore, normal esophageal tissues were more difficult to obtain.

The content of lactic acid in the cell fragment and culture supernatant of the sh-MCT1 group was determined and compared with that of the sh-NC group, but the results were disappointing. Different groups tended to have equal amounts of lactic acid as shown by the results (data not shown). We should have determined the pH values of both groups rather than the content of certain acids, but the pH meter in our labo-ratory was not available.

Surgical procedures are an important treatment means for early stage patients. Chemotherapy and radiotherapy are critical for patients who cannot tolerate surgery or who are at advanced stages (1). Concurrent radio-chemotherapy (CRT) brings significant benefits to non-surgical patients.

Chemotherapy resistance and radioresistance are principal challenges for malignant tumor treatment. MCT1 has been investigated in studies of cisplatin resistance in ovarian cancer and radiosensitivity in small cell lung cancer (SCLC) (35,36). Since the research direction of our team is the radioresistance and radiosensitization of esophageal cancer, the latent role of MCT1 in the radiotherapy of ESCC will be an active area of research for us. We plan to devote further research to this topic.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XueC and XuanC had equal contribution in conducting the experiments and writing the manuscript. YC was responsible for the design of the study and served as academic advisor throughout the process. FL was responsible for the acquisition and scoring of the IHC slides. QY, KZ, WZ, SG, YW and SM acquired the follow-up data and calculated the OS (overall survival) and PFS (progression-free survival) of 103 patients. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The research protocol of the present study was approved by the Ethics Committee of Qilu Hospital, and all patients signed written informed consents before enrollment. Patient information was anonymized and unidentifiable prior to analysis.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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