

# Mitochondrial calcium uniporter promotes cell proliferation and migration in esophageal cancer

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**Abstract.** Increasing evidence has suggested that mitochondrial calcium uniporter (MCU) is involved in various types of cancer. However, its functions remain unclear in esophageal cancer. The aim of the present study was to explore its abnormal expression and clinical implications in esophageal cancer. A total of 110 patients with esophageal cancer were enrolled in the study. Western blotting was performed to examine the protein expression levels of MCU in 8 pairs of esophageal cancer and adjacent normal tissues. Using immunohistochemistry, a total of 110 esophageal cancer specimens were analyzed to identify the association between MCU expression and clinicopathological features of patients with esophageal cancer. Furthermore, immunofluorescence of MCU was performed. Pearson's correlation analysis was performed between MCU and hypoxia inducible factor (HIF)-1 $\alpha$ /VEGF/E-cadherin/Vimentin expression based on western blotting. After KYSE-150 and TE-1 cells were treated with the MCU agonist Spermine and a small interfering RNA against MCU (si-MCU), a series of functional assays were performed, including Cell Counting Kit-8, colony formation and Transwell assays. The results revealed that, compared with adjacent normal tissues, MCU was highly expressed in esophageal cancer tissues. MCU expression was significantly associated with depth of invasion, lymph node metastasis, TNM stage and distant metastasis. Moreover, MCU was significantly correlated with HIF-1 $\alpha$ /VEGF/E-cadherin/Vimentin in esophageal cancer tissues. MCU overexpression promoted

VEGF, MMP2, Vimentin and N-cadherin expression, while it inhibited E-cadherin expression in KYSE-150 and TE-1 cells, and opposite results were observed after transfection with si-MCU. Furthermore, MCU overexpression accelerated the proliferation and migration of KYSE-150 and TE-1 cells. Thus, the current findings suggested that high MCU expression may participate in cell proliferation, migration and epithelial-mesenchymal transition in esophageal cancer.

## Introduction

Esophageal cancer is one of the most aggressive types of cancer, with high morbidity and mortality worldwide (1). There are two most common histological subtypes: Squamous cell carcinoma and adenocarcinoma. Despite marked improvements of therapies, the overall survival rate of patients with esophageal cancer remains unsatisfactory (2). The poor prognosis of esophageal cancer is attributed to early metastasis (3). Although it has been determined that certain tumor suppressors and oncogenes serve a key role in the occurrence and development of esophageal cancer, it is urgent to identify molecular markers to improve the early diagnosis and prognosis of esophageal cancer (3).

Mitochondrial calcium uniporter (MCU) has been identified as a channel responsible for mitochondrial Ca<sup>2+</sup> absorption, which may control cell energy metabolism, ROS production and programmed cell death (4). All cancer tissues exhibit moderate to strong MCU immunostaining (5). Increasing evidence has suggested that MCU serves an important role in cancer metastasis. For example, MCU is highly expressed in metastatic breast cancer and induces cancer metastasis through the Warburg effect (6). MCU-knockdown significantly suppresses cell migration and invasion in triple-negative breast cancer xenografts and decreases tumor growth, lymph node infiltration and lung metastasis (7). MCU is closely associated with metastasis and poor prognosis of patients with hepatocellular carcinoma (8). MCU expression is upregulated in pancreatic cancer tissues compared with in normal tissues, which may be partly mediated by the HINT2 protein (9). Furthermore, MCU is involved in various cellular biological processes. For example, MCU loss hinders cell cycle progression and proliferation (10). However, the expression

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and clinical implications of MCU in esophageal cancer remain unclear. In the present study, the expression levels of MCU were detected in 110 esophageal cancer tissues and its role in esophageal cancer was analyzed.

## Materials and methods

**Bioinformatics analysis.** The expression levels of MCU were assessed between tumor and normal samples in various types of cancer via Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia2.cancer-pku.cn/#index>). MCU expression between esophageal cancer samples (n=182) and normal tissues (n=286) was analyzed in The Cancer Genome Atlas (TCGA; <https://portal.gdc.cancer.gov/>) normal and GTEx datasets (<http://www.gtexportal.org/home/>). The differences in MCU expression among different tumor stages were compared in TCGA-Esophageal carcinoma (ESCA) dataset ([https://portal.gdc.cancer.gov/projects?filters=%7B%22op%22%3A%22and%22%2C%22content%22%3A%5B%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22projects.project\\_id%22%2C%22value%22%3A%5B%22TCGA-ESCA%22%5D%7D%7D%5D%7D](https://portal.gdc.cancer.gov/projects?filters=%7B%22op%22%3A%22and%22%2C%22content%22%3A%5B%7B%22op%22%3A%22in%22%2C%22content%22%3A%7B%22field%22%3A%22projects.project_id%22%2C%22value%22%3A%5B%22TCGA-ESCA%22%5D%7D%7D%5D%7D)).

**Patients and specimens.** Patients with esophageal cancer who underwent esophagectomy were enrolled in the General Hospital of Ningxia Medical University (Yinchuan, China) between January 2016 to December 2017. The inclusion criteria were as follows: i) Patients were diagnosed with esophageal cancer by at least two experienced pathologists according to histopathology examination; and ii) patients did not receive chemotherapy or radiation therapy before surgery. Finally, a total of 110 patients (mean age, 58.6 years old and age range, 42.6-75.3 years old) with esophageal cancer were included in the study. All tumor and adjacent normal tissues were collected from patients and the distance of the normal tissues was at least 5 cm from the tumor tissues. All specimens were immediately transferred to liquid nitrogen after surgery, and stored at -80°C until use. Clinical information including sex, age, depth of invasion, lymph node metastasis, TNM stage and distant metastasis was also obtained. The present study strictly followed the guidelines of the Declaration of Helsinki. All patients provided written informed consent. The study was approved by the ethics committee of General Hospital of Ningxia Medical University (approval no. 2016049).

**Hematoxylin and eosin (H&E) staining.** Esophageal cancer and normal tissue specimens were fixed by 4% paraformaldehyde solution at 4°C for 24 h. After being embedded in paraffin, the tissues were cut into 20- $\mu$ m pieces. The sections were stained with 0.5% hematoxylin for 5 min at 37°C and 0.5% eosin solution for 5 min at 37°C. Neutral gum was utilized for sealing and images were observed under an light microscope (Olympus Corporation) (magnification, x200).

**Immunohistochemistry.** Fresh tissue specimens were fixed with 4% paraformaldehyde overnight at 4°C and dehydrated using different concentrations of alcohol. The specimen was incubated in a 1:1 mixture of 100% alcohol and xylene for 30 min at room temperature and then transferred to xylene

for 30 min at 37°C. Subsequently, the tissues were embedded in paraffin, and the tissue sections were cut to a thickness of 4  $\mu$ m. The sections were placed in a 60°C incubator for 90 min and then quickly placed in xylene I for 15 min and xylene II for 15 min at 37°C. Subsequently, the tissue sections were placed in absolute ethanol I, absolute ethanol II, 95, 90, 80, 70 and 50% ethanol for 5 min each at 37°C, followed by deionized water for 5 min. Antigen retrieval was performed using the high-pressure method (11). After washing 3 times with 1X PBS, the sections were incubated with 3% hydrogen peroxide at 37°C for 20 min to eliminate endogenous peroxidase activity. After washing again, the sections were blocked with 2% BSA (Beyotime Institute of Biotechnology) at 37°C for 2 h. The tissue sections were incubated with primary antibodies including anti-MCU (1:100; cat. no. sc-515930; Santa Cruz Biotechnology, Inc.), anti-VEGF (1:100; cat. no. 26381-1-AP; ProteinTech Group, Inc.), anti-Vimentin (1:150; cat. no. ab92547; Abcam), anti-E-cadherin (1:200; cat. no. ab40772; Abcam), anti-MMP2 (1:100; cat. no. 10373-2-AP; ProteinTech Group, Inc.), and anti-N-cadherin (1:100; cat. no. ab18203; Abcam) overnight at 4°C, followed by secondary antibody (1:200; cat. no. ab150077; Abcam) incubation at 37°C for 1 h. The sections were stained with DAB for 5 min and hematoxylin for 2 min at 37°C. Subsequently, the sections were placed in hydrochloric acid monoethanol for color separation for 30 sec and turned back to blue with 1X PBS for 2 min. After dehydration and transparency, neutral gum was added to the tissue sections, followed by air drying.

**Immunohistochemistry scoring assessment.** Double-blind readings were performed by two experienced pathologists. The positive expression of target proteins was the appearance of brown particles on the cell membrane and/or cytoplasm. In the present study, semi-quantitative results were used to evaluate the percentage of positive cells and staining intensity, respectively (12). The number of positively stained cells was observed under 5 high-power fields using a light microscope (Olympus Corporation) (magnification, x200) on each slide, and then the percentage of positive cells was counted and scored as follows: 0, <5% Positive cells; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Staining intensity was scored as follows: 0, Colorless; 1, light yellow; 2, claybank; and 3, sepia. These two scores were multiplied to obtain the positive grade: 0, Negative (-); 1-4, weak positive (+); 5-8, positive (++); and 9-12, strong positive (+++). The optical density of target proteins was measured using ImageJ software v. 1.48 (National Institutes of Health).

**Western blot analysis.** Tissues and cells were lysed by RIPA cell lysate (Beyotime Institute of Biotechnology) plus protease inhibitor PMSF and phosphatase inhibitor cocktail for 40 min on ice, followed by centrifugation at 12,000 x g for 15 min at 4°C. Subsequently, the supernatant was harvested and stored at -80°C. The BCA method was used to measure the total protein concentration according to the instructions of the BCA™ Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (30  $\mu$ g) was loaded per lane and protein samples were separated via 12% SDS-PAGE and then transferred to a PVDF membrane (IPVH00010; EMD Millipore). The membrane

was blocked with 5% skimmed milk at room temperature for 2 h and was then incubated with primary antibodies including anti-MCU (1:500; cat. no. sc-515930; Santa Cruz Biotechnology, Inc.), anti-VEGF (1:1,000; cat. no. 26381-1-AP; ProteinTech Group, Inc.), anti-hypoxia inducible factor (HIF)-1 $\alpha$  (1:1,000; cat. no. 20960-1-AP; ProteinTech Group, Inc.) anti-Vimentin (1:2,000; cat. no. ab92547; Abcam), anti-E-cadherin (1:1,000; cat. no. ab40772; Abcam), anti-MMP2 (1:1,000; cat. no. ab181286; Abcam), anti-N-cadherin (1:1,000; cat. no. ab20760; Abcam) and anti- $\beta$ -actin (1:2,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.) at 4°C overnight. After washing the membrane, the membrane was incubated with a horseradish peroxidase (HRP) secondary antibody (1:2,000; cat. no. ab7090; Abcam) at room temperature for 2 h. The protein band was visualized using BeyoECL Plus (cat. no. KGP1121; Nanjing KeyGen Biotech Co., Ltd.). ImageJ software v.1.48 (National Institutes of Health) was used for quantifying the expression of target proteins.

**Immunofluorescence.** In brief, the tissue sections were incubated with unconjugated-labeled primary antibodies including anti-MCU (1:100; cat. no. sc-515930; Santa Cruz Biotechnology, Inc.), anti-E-cadherin (1:100; cat. no. ab40772; Abcam) and anti-Vimentin (1:100; cat. no. ab92547; Abcam) overnight at 4°C, followed by Alexa Fluor® 488-conjugated (1:100; cat. no. ZF-0512; OriGene Technologies, Inc.) and Alexa Fluor® 594-conjugated (1:100; cat. no. ZF-0513; OriGene Technologies, Inc.) secondary antibodies at room temperature for 2 h. For nuclear counterstaining, cells were incubated with DAPI (1:100; cat. no. ab104139; Abcam) for 5 min at room temperature. The images were acquired by confocal microscopy (General Electric Company) (magnification, x200). The results were determined using ImageJ software v.1.48 (National Institutes of Health).

**Cell culture.** Two human esophageal cancer KYSE-150 and TE-1 cell lines (American Type Culture Collection) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific Inc.), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO<sub>2</sub>.

**Transfection.** Cells were seeded onto a 6-well plate. KYSE-150 and TE-1 cells were treated with different concentrations (10, 20, 30, 50, 70, 100 and 200  $\mu$ M) of the MCU agonist Spermine (cat. no. 18041; Cayman Chemical Company) at 37°C for 48 h. A small interfering RNA (siRNA) against MCU (si-MCU) was designed as follows: si-MCU#1 sense, 5'-CAUAAAGGA GCCAAAAGUCA-3' and antisense, 5'-ACUUUUUGG CUCCUUUAUGGA-3'; si-MCU#2 sense, 5'-GGGAAUUGA CAGAGUUGCU-3' and antisense, 5'-AGCAACUCUGUCAAUUC-3'. Meanwhile, the sequences of the siRNA negative control (si-NC; scrambled RNA) were as follows: sense, 5'-GCCUAAGAACGACAAAUCA-3' and antisense, 5'-UGAUUUGUCGUUCUUAGGC-3'. A total of 5 nM of si-MCU or si-NC was transfected into cells via Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific Inc.) at room temperature for 48 h. After 48 h, western blotting was used to examine the transfection effects.

**Colony formation assay.** Cells of each group were collected 24 h after transfection and were inoculated in 35-mm culture dishes (500-1,000 cells/dish). These cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 14 days. The culture medium was changed every 4-5 days. Following rinsing with 1X PBS for 3 times, the cells were fixed with methanol for 10 min at room temperature and stained with 0.5% crystal violet for 20 min at room temperature. Following rinsing and drying, the colonies were photographed and counted. A total of 50 cells were considered as a colony.

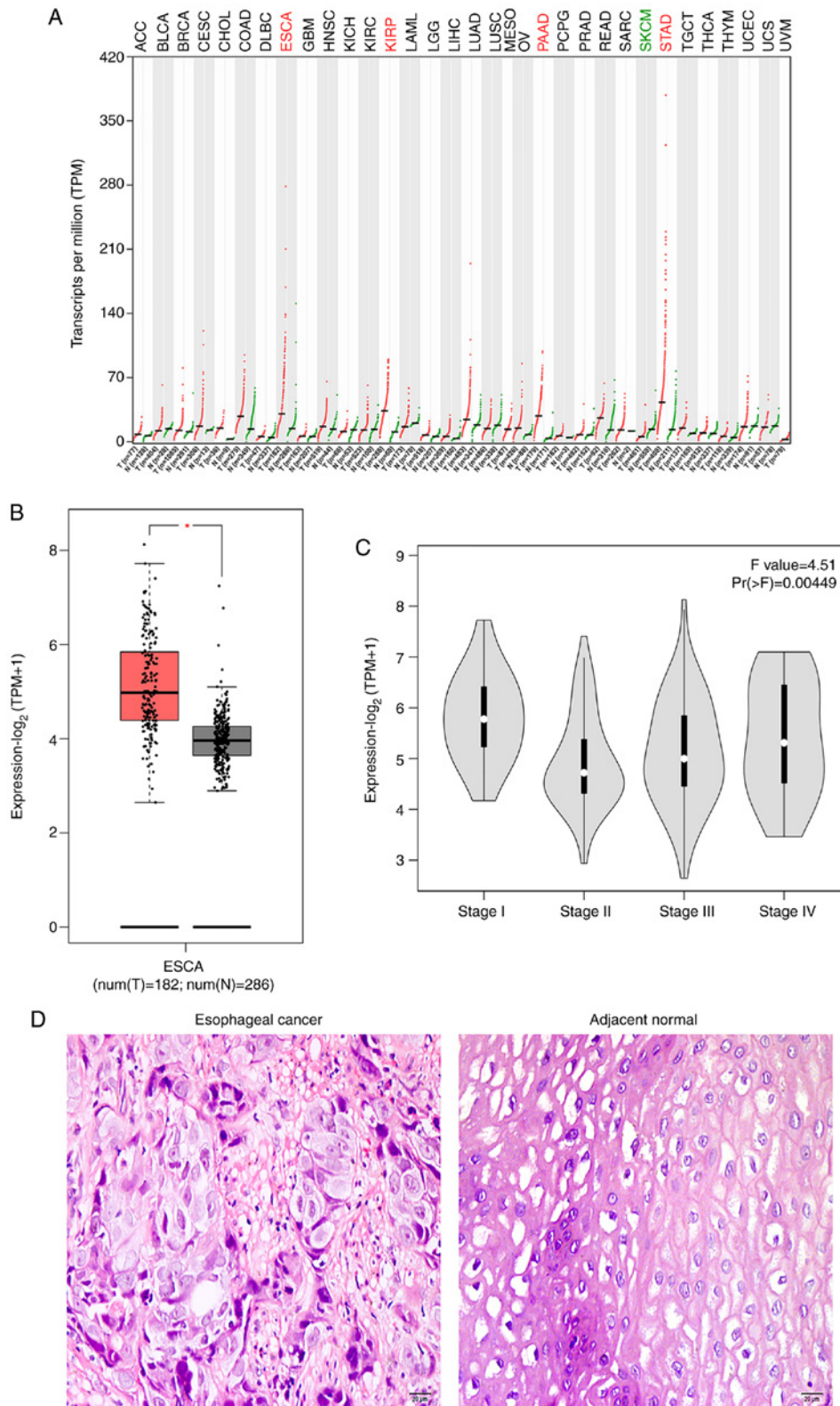
**Cell Counting Kit-8 (CCK-8) assay.** Cells were seeded into a 96-well plate. After 48 h from transfection, cell viability was determined using the CCK-8 reagent (Dojindo Molecular Technologies, Inc.) for 1 h. The absorbance at 450 nm was assessed utilizing a multiscan spectrum.

**Transwell assay.** After 24 h from transfection, cells were starved with serum-free RPMI-1640 medium for 6-8 h. The digested cells were washed twice with serum-free RPMI-1640 medium and were then seeded in the upper chamber of a 24-well Transwell insert (1.5x10<sup>9</sup> cells/well). A total of 600  $\mu$ l RPMI-1640 medium containing 20% FBS was added to the lower chamber. The transfected cells were cultured at 37°C with 5% CO<sub>2</sub> for 24 h. Afterwards, the cells were fixed using 4% paraformaldehyde for 15 min at 37°C and stained using Giemsa for 30 min at 37°C. Migratory cells in lower chamber were observed under a light microscope (Olympus Corporation) (magnification, x200; scale bar 50  $\mu$ m).

**Statistical analysis.** All statistical analysis was performed using SPSS 19.0 software (IBM Corp.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). The results were expressed as the mean  $\pm$  SD from at least three independent experiments. The comparisons between two groups were analyzed using unpaired Student's t-test and one-way analysis of variance followed by Tukey's post-hoc test was performed for multiple comparisons. The association between MCU expression and clinical traits was analyzed using the  $\chi^2$  test. Furthermore, Pearson's correlation analysis was used to analyze the correlation between MCU expression and other proteins. P<0.05 and correlation coefficient  $|r|>0.3$  were considered to indicate a statistically significant difference.

## Results

**MCU expression is upregulated in esophageal cancer.** According to GEPIA, MCU was highly expressed in esophageal cancer, kidney renal papillary cell carcinoma, pancreatic adenocarcinoma and stomach adenocarcinoma among various types of cancer (Fig. 1A). Box plots visualized the differences in MCU expression between esophageal cancer samples (n=182) and normal tissues (n=286) from TCGA in Fig. 1B (P<0.05). Additionally, there was a significant difference in MCU expression among different stages of esophageal cancer (Fig. 1C). A total of 110 patients with esophageal cancer were included in the present study. Fig. 1D shows the representative images of H&E staining results of esophageal cancer tissues and the corresponding adjacent normal tissues. Compared with normal epithelium cells of esophageal tissues, tumor cells



**Figure 1.** MCU upregulation in esophageal cancer using Gene Expression Profiling Interactive Analysis. (A) Patterns of MCU expression in various types of cancer. (B) Box plots showing the upregulation of MCU expression in esophageal cancer.  $P < 0.05$ . (C) Violin diagram showing the difference in MCU expression among different stages of esophageal cancer. (D) Representative images of hematoxylin and eosin staining results of esophageal cancer and corresponding adjacent normal tissues. Scale bar,  $20 \mu\text{m}$ ; magnification,  $\times 200$ . Red represented the upregulation of MCU expression in this cancer type while green represented the downregulation of MCU expression in this cancer type. MCU, mitochondrial calcium uniporter; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

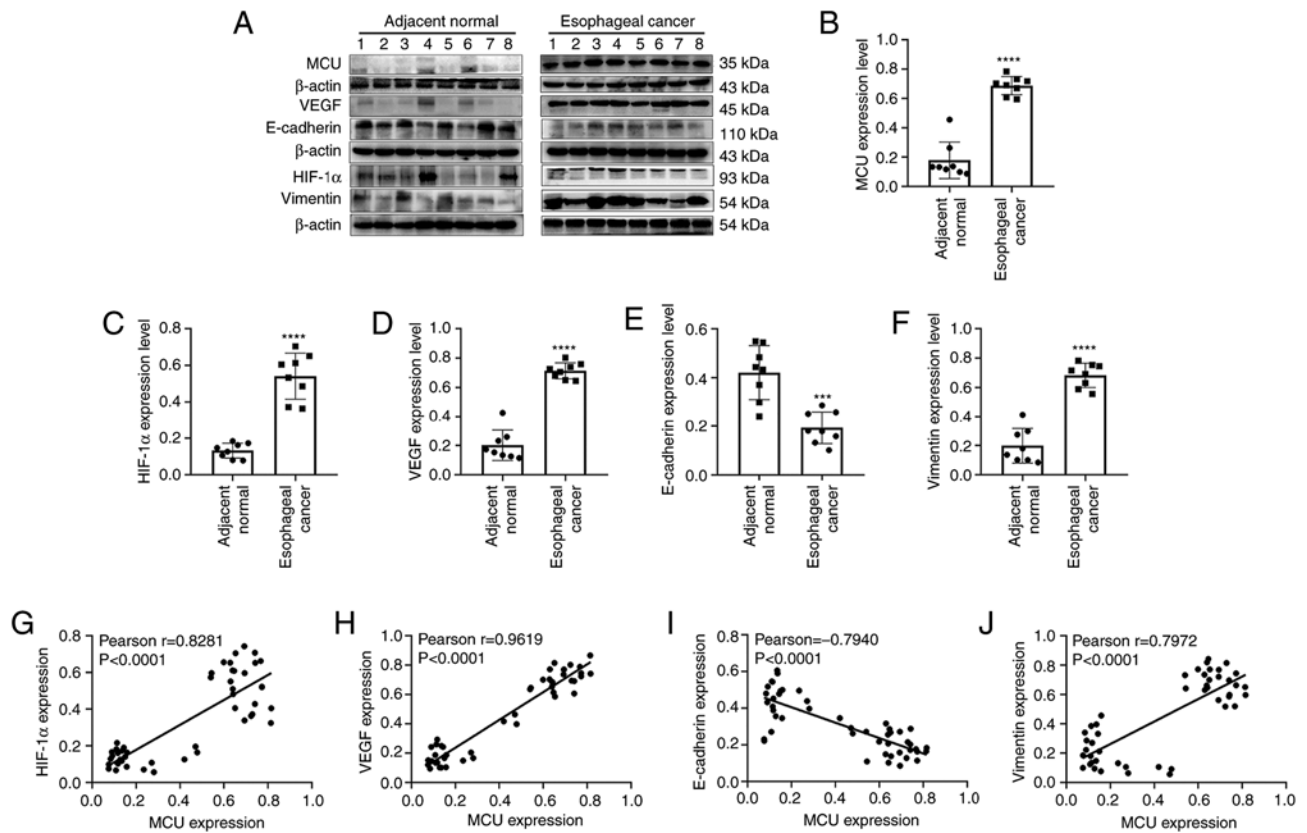


Figure 2. Western blotting results showing the protein expression levels of MCU, HIF-1 $\alpha$ , VEGF, E-cadherin and Vimentin in esophageal cancer and adjacent normal tissues. (A) Representative images of western blots. Protein expression levels of (B) MCU, (C) HIF-1 $\alpha$ , (D) VEGF, (E) E-cadherin and (F) Vimentin between esophageal cancer and adjacent normal tissues. Correlation between MCU and (G) HIF-1 $\alpha$ , (H) VEGF, (I) E-cadherin and (J) Vimentin expression. Each experiment was repeated three times. \*\*\*P<0.001 and \*\*\*\*P<0.0001. MCU, mitochondrial calcium uniporter; HIF, hypoxia inducible factor.

had large nuclei and were in tight contact with neighbor cells. A total of 8 pairs of esophageal cancer and adjacent normal tissues were randomly selected for western blot analysis. As expected, MCU protein expression was significantly higher in esophageal cancer tissues compared with in adjacent normal tissues (Fig. 2A and B).

*MCU expression is significantly correlated with HIF-1 $\alpha$ /VEGF/E-cadherin/Vimentin expression in esophageal cancer.* Tumor metastasis is the main cause of esophageal cancer mortality (13). Epithelial-mesenchymal transition (EMT) is associated with downregulation of E-cadherin and upregulation of vimentin expression, which contributes to tumor invasion and metastasis (14). Meanwhile, the HIF-1 $\alpha$ /VEGF signaling pathway, which can mediate the EMT process, has been considered a key target for treating cancer metastasis (14). Thus, the expression levels of HIF-1 $\alpha$ , VEGF, E-cadherin and Vimentin were detected between 8 pairs of esophageal cancer tissues and adjacent normal tissues using western blotting. HIF-1 $\alpha$  (Fig. 2C) and VEGF (Fig. 2D) protein expression was significantly higher in esophageal cancer compared with in adjacent normal tissues. Additionally, low E-cadherin expression was observed in esophageal cancer tissues (Fig. 2E), while vimentin had higher expression levels in esophageal cancer than in adjacent normal tissues (Fig. 2F). Correlation analysis was then performed, revealing that there were positive correlations between MCU expression and HIF-1 $\alpha$  (Pearson  $r=0.8281$ ;  $P<0.0001$ ; Fig. 2G)

and VEGF (Pearson  $r=0.9619$ ;  $P<0.0001$ ; Fig. 2H) expression. Additionally, a negative correlation was observed between MCU and E-cadherin expression in esophageal cancer tissues (Pearson  $r=-0.7940$ ;  $P<0.0001$ ; Fig. 2I). Furthermore, MCU was positively correlated with Vimentin (Pearson  $r=0.7972$ ;  $P<0.0001$ ; Fig. 2J). Subsequently, immunofluorescence analysis of MCU, E-cadherin and Vimentin was also performed in esophageal cancer and adjacent normal tissues. Consistent with the western blot results, the immunofluorescence results revealed that MCU protein expression was significantly higher and E-cadherin protein expression was significantly lower in esophageal cancer compared with in adjacent normal tissues (Fig. 3A and B). As shown in Fig. 3C and D, both MCU and Vimentin exhibited higher expression levels in esophageal cancer than in adjacent normal tissues.

*Association between MCU expression and clinicopathological features of patients with esophageal cancer.* The expression and localization of MCU were detected by immunohistochemistry in 110 patients with esophageal cancer. Among 110 esophageal cancer specimens, 88 had positive expression of MCU (including weak positive, positive or strong positive expression) and others had negative expression of MCU (Table I). The association between MCU expression and clinical characteristics, including sex, age, depth of invasion, lymph node metastasis, TNM stage and distant metastasis, was analyzed. As shown in Table I, MCU expression was significantly associated with depth of invasion ( $P=0.031$ ),

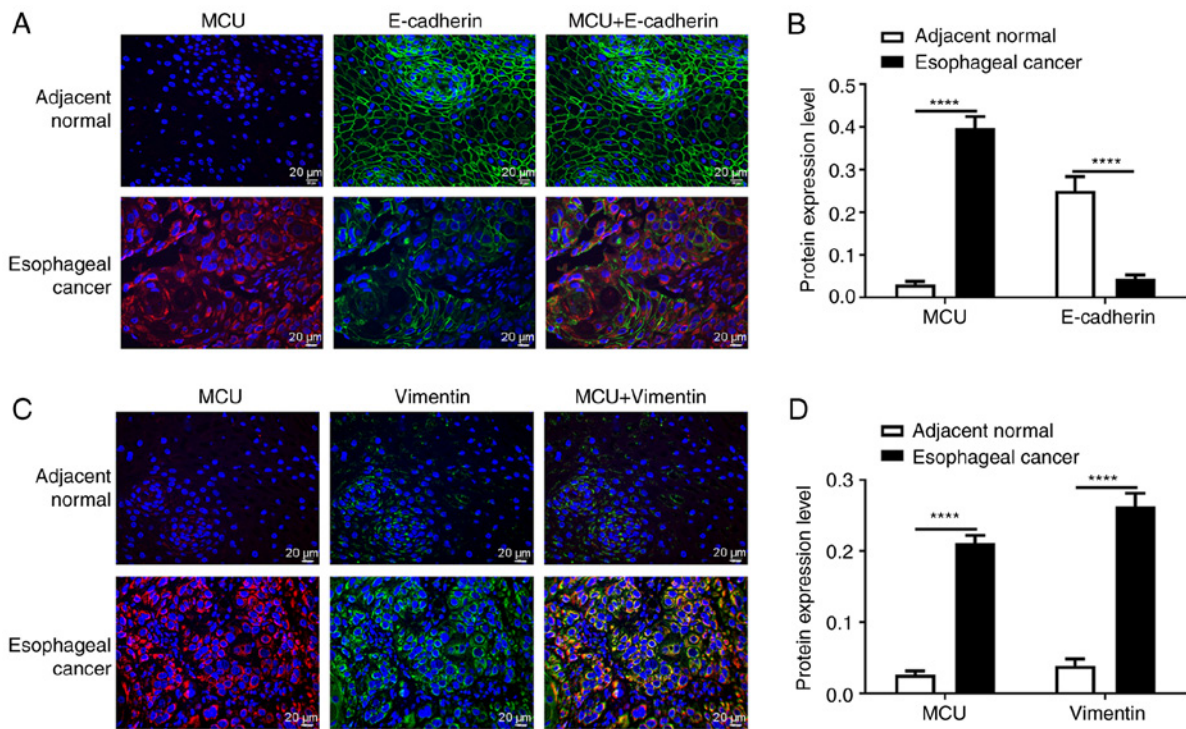


Figure 3. Immunofluorescence results of MCU, E-cadherin and Vimentin expression in esophageal cancer and adjacent normal tissues. (A) Representative images of MCU and E-cadherin expression. (B) Expression levels of MCU and E-cadherin between esophageal cancer and adjacent normal tissues. (C) Representative images of MCU and Vimentin expression. (D) Expression levels of MCU and Vimentin between esophageal cancer and adjacent normal tissues. Red represents MCU and green represents E-cadherin or Vimentin. Scale bar, 20 μm; magnification, x200. \*\*\*\*P<0.0001. MCU, mitochondrial calcium uniporter.

lymph node metastasis ( $P=0.027$ ), TNM stage ( $P=0.036$ ) and distant metastasis ( $P=0.008$ ). However, there was no association between MCU expression and sex ( $P=0.332$ ) and age ( $P=0.381$ ) (Table I). Furthermore, MCU was mainly expressed in the cytoplasm. Representative results of MCU staining are shown in Fig. 4A. Significantly higher MCU expression was detected in esophageal cancer tissues compared with in adjacent normal tissues (Fig. 4B).

*Immunohistochemistry results of VEGF, Vimentin, E-cadherin, MMP2 and N-cadherin in esophageal cancer.* As aforementioned, MCU expression was significantly correlated with VEGF and EMT-associated proteins. Hence, the protein expression levels of VEGF, Vimentin, E-cadherin, MMP2 and N-cadherin between esophageal cancer and adjacent normal tissues were further examined by immunohistochemistry. Consistent with the aforementioned results, higher expression levels of VEGF and Vimentin were detected in esophageal cancer compared with in adjacent normal tissues (Fig. 4A and B). Furthermore, E-cadherin expression was significantly lower in esophageal cancer than in adjacent normal tissues, while MMP2 and N-cadherin protein expression was significantly upregulated in esophageal cancer tissues compared with in adjacent normal tissues (Fig. 4A and B), indicating that these proteins may be involved in the development of esophageal cancer.

*MCU promotes VEGF, MMP2, Vimentin and N-cadherin, and inhibits E-cadherin in esophageal cancer cells.* Western blotting was performed to validate the transfection effect of si-MCU in KYSE-150 and TE-1 cells. As shown in

Fig. 5A and B, MCU protein expression was significantly suppressed in KYSE-150 and TE-1 cells. After overexpression of MCU using Spermine, VEGF, Vimentin and N-cadherin expression was significantly increased in KYSE-150 cells (Fig. 5C-H). MCU overexpression did not significantly alter MMP2 expression. However, their expression levels were significantly suppressed and E-cadherin expression was significantly promoted in KYSE-150 cells after transfection with si-MCU (Fig. 5C-I). Similar results of MCU, VEGF, MMP2, Vimentin, N-cadherin and E-cadherin expression were obtained in TE-1 cells treated with Spermine or transfected with si-MCU (Fig. 5C and J-O).

*MCU accelerates the proliferation and migration of esophageal cancer cells.* The cellular function of MCU in esophageal cancer was further explored. CCK-8 results indicated that the viability of KYSE-150 and TE-1 cells was significantly inhibited with Spermine in a concentration-dependent manner (Fig. 6A and B). The concentration of 30 μm/ml Spermine was chosen as the optimal concentration for further analysis. As shown in Fig. 6C-E, MCU overexpression significantly promoted the proliferation of KYSE-150 and TE-1 cells, while opposite results were observed after transfection with si-MCU. Transwell assay results demonstrated that the migration of KYSE-150 and TE-1 cells was significantly promoted by MCU overexpression, while it was significantly suppressed after si-MCU transfection (Fig. 6F-H).

## Discussion

The development of esophageal cancer is a complex process involving multiple steps and multiple factors. Despite surgical

Table I. Association between clinicopathological parameters and MCU expression in 110 patients with esophageal cancer.

Clinical parameters	Total, n	MCU expression <sup>e</sup> , n		$\chi^2$	P-value
		Positive (n=88)	Negative (n=22)		
Sex					
Male	65	54	11	0.940	0.332
Female	45	34	11		
Age, years					
<60	66	51	15	0.767	0.381
≥60	44	37	7		
Depth of invasion					
T1/T2	42	38	4	4.660	0.031 <sup>a</sup>
T3/T4	68	50	18		
Lymph node metastasis					
N0	38	26	12	4.865	0.027 <sup>a</sup>
N1/N2/N3	72	62	10		
TNM stage					
I-II	39	27	12	4.380	0.036 <sup>a</sup>
III-IV	71	61	10		
Distant metastasis					
Absent	43	29	14	6.959	0.008 <sup>b</sup>
Present	67	59	8		

<sup>a</sup>P<0.05 and <sup>b</sup>P<0.01. <sup>c</sup>Patients were divided into positive and negative expression groups, where the positive expression group included weak positive, positive and strong positive expression. MCU, mitochondrial calcium uniporter.

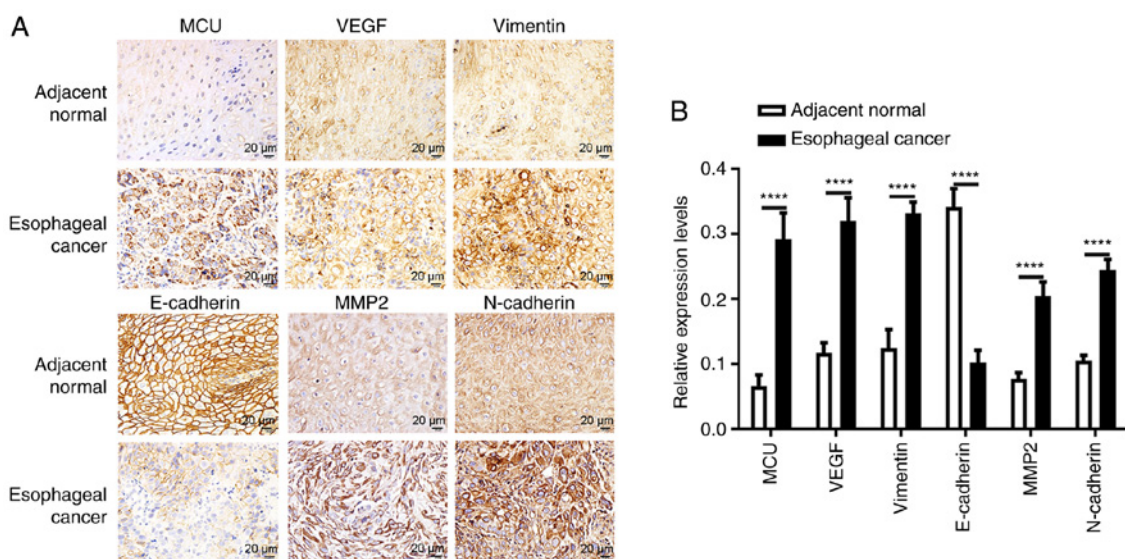


Figure 4. Immunohistochemistry results of MCU, VEGF, Vimentin, E-cadherin, MMP2 and N-cadherin in esophageal cancer and adjacent normal tissues. (A) Representative images of immunohistochemistry results. (B) Expression levels of MCU, VEGF, Vimentin, E-cadherin, MMP2 and N-cadherin between esophageal cancer and adjacent normal tissues. Scale bar, 20 μm; magnification, x200. \*\*\*\*P<0.0001. MCU, mitochondrial calcium uniporter.

resection and other novel treatments, patients with esophageal cancer are usually diagnosed in the middle and late stages, leading to a high mortality and poor prognosis (15). Moreover, lymph node metastasis is an important event for esophageal cancer, since it may contribute to a high recurrence rate (16,17). The present study identified that MCU was significantly

highly expressed in esophageal cancer. Its high expression promoted the proliferation, migration and EMT of esophageal cancer cells. Thus, high MCU expression may contribute to esophageal cancer metastasis.

The present study analyzed MCU expression in patients with esophageal cancer by western blotting, immunohistochemistry

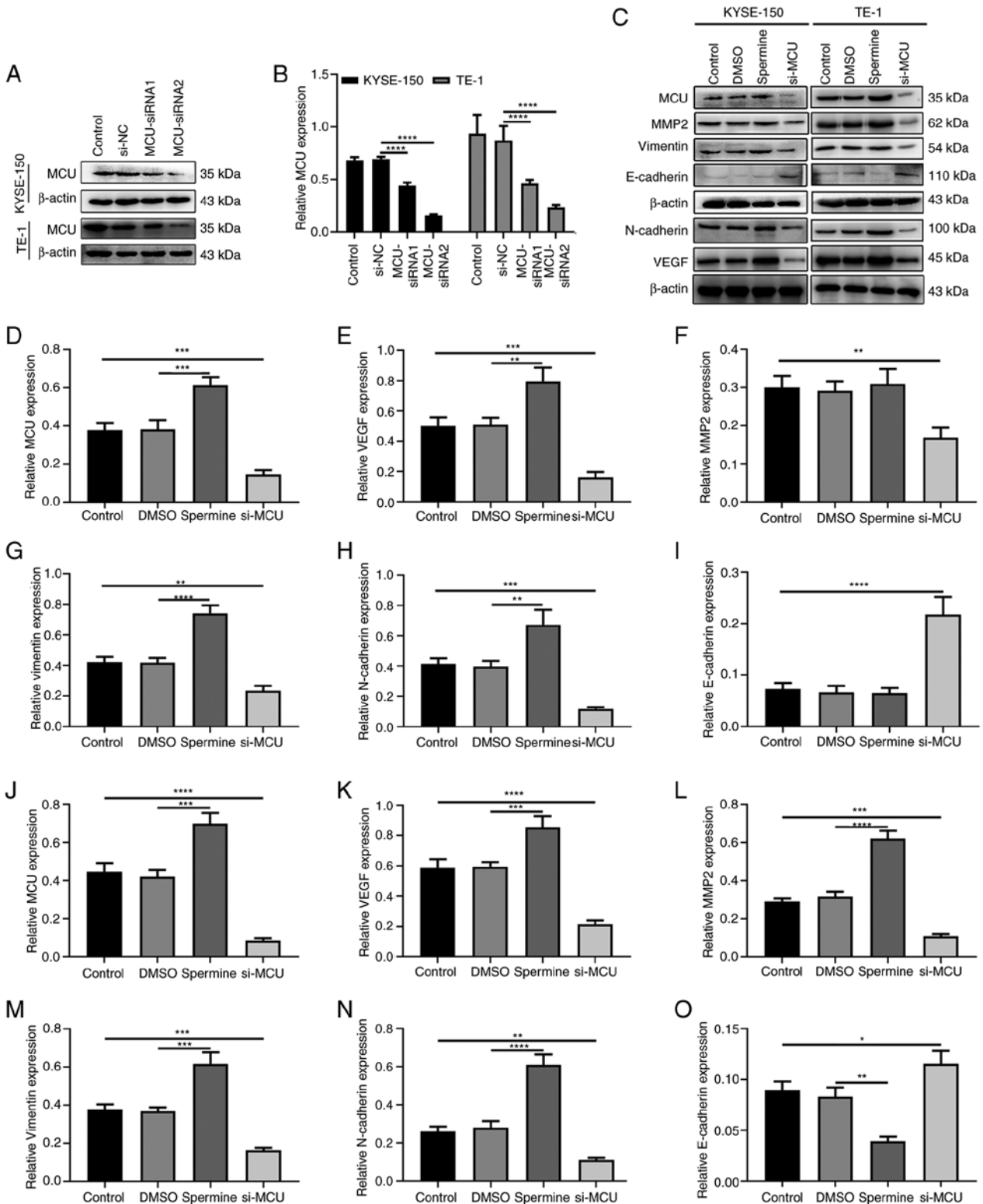


Figure 5. MCU promotes VEGF, MMP2, Vimentin and N-cadherin expression, and inhibits E-cadherin expression in esophageal cancer cells. (A and B) Western blotting was used to validate the effect of si-MCU transfection in KYSE-150 and TE-1 cells. (C) Representative images of western blotting for the protein expression levels of VEGF, MMP2, Vimentin, N-cadherin and E-cadherin in KYSE-150 and TE-1 cells treated with Spermine or transfected with si-MCU. Quantification of protein expression levels of (D) MCU, (E) VEGF, (F) MMP2, (G) Vimentin, (H) N-cadherin and (I) E-cadherin in KYSE-150 cells treated with Spermine or si-MCU. Quantification of protein expression levels of (J) MCU, (K) VEGF, (L) MMP2, (M) Vimentin, (N) N-cadherin and (O) E-cadherin in TE-1 cells treated with Spermine or si-MCU.  $\beta$ -actin was used as a reference control. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . MCU, mitochondrial calcium uniporter; si/siRNA, small interfering RNA; NC, negative control.

and immunofluorescence. Moreover, the association between MCU expression and clinical characteristics was analyzed. The current data suggested that MCU expression was higher

in esophageal cancer tissues compared with normal tissues. In 110 cases, there were significant associations between MCU expression and TNM stage and lymph node metastasis. It



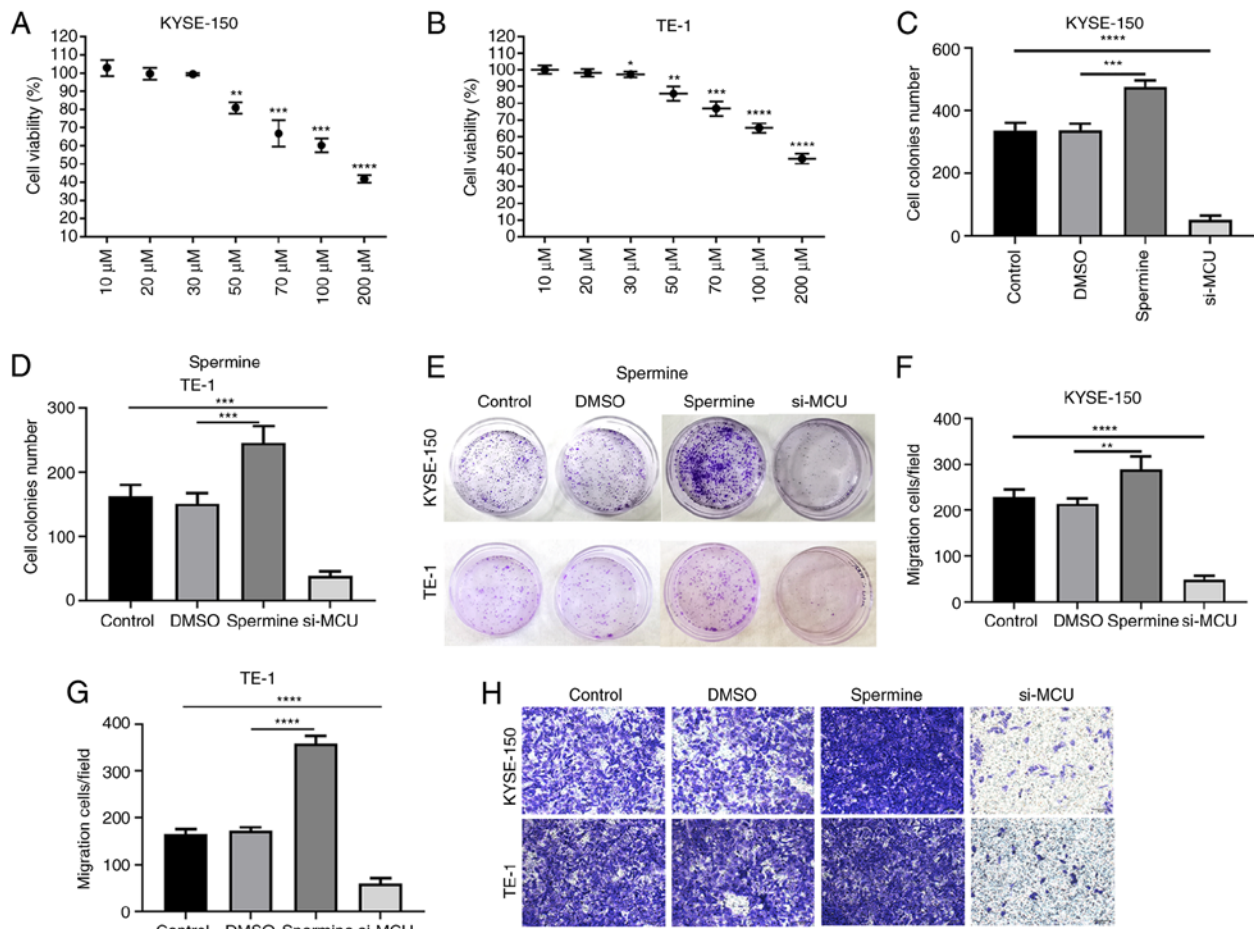


Figure 6. MCU accelerates the proliferation and migration of esophageal cancer cells. Cell viability was detected in (A) KYSE-150 and (B) TE-1 cells treated with different concentrations of Spermine using Cell Counting Kit-8 assay. Colony formation assay was used to examine the proliferative capacity of KYSE-150 and TE-1 cells treated with Spermine or si-MCU. The number of (C) KYSE-150 and (D) TE-1 cell colonies was quantified, and (E) representative images of colony formation assay. Transwell assay was performed to assess the migratory ability of KYSE-150 and TE-1 cells after treatment with Spermine or si-MCU. The number of migrated (F) KYSE-150 and (G) TE-1 cells was quantified, and (H) representative images of Transwell assay. Magnification, x200 and scale bar, 50  $\mu\text{m}$ . \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . MCU, mitochondrial calcium uniporter; si, small interfering RNA.

has been identified that the prognosis of esophageal cancer is greatly affected by lymph node metastasis, which is associated with poor survival (18). The current results indicated that high MCU expression was associated with the invasiveness of esophageal cancer, thereby serving an important role in the metastasis of esophageal cancer.

According to the present western blotting results, MCU expression was significantly correlated with HIF-1 $\alpha$  and VEGF expression, and with the EMT process in esophageal cancer. In the present study, a positive correlation was identified between MCU and HIF-1 $\alpha$  expression. Similar results were observed in breast cancer tissues (7). Furthermore, silencing MCU can inhibit HIF-1 $\alpha$  expression in colon cancer and triple-negative breast cancer (7,19). These findings indicated that HIF-1 $\alpha$  may be regulated by MCU. In the current study, it was revealed that MCU promoted VEGF, MMP2, Vimentin and N-cadherin expression, and inhibited E-cadherin expression in esophageal cancer cells. However, further studies need to be performed. Tumor metastasis is the main cause of death for most patients with esophageal cancer (20). HIF-1 $\alpha$  activation is one of the most important mechanisms that promotes metastasis and increases tumor aggressiveness (21-23). Additionally, high VEGF expression was found in esophageal cancer tissues,

which may serve a major angiogenic role in esophageal cancer. Several meta-analysis studies have revealed that high VEGF expression is associated with poor overall survival in patients with esophageal cancer (24,25). Furthermore, it has been confirmed that VEGF is a downstream target of HIF-1 $\alpha$  in esophageal cancer (26). EMT is one of the main mechanisms for inducing tumor invasion and metastasis (27,28). EMT is a process in which epithelial cells lose their polarity and acquire a mesenchymal phenotype (24,25). Hypoxia induced by HIF-1 $\alpha$  is an important microenvironment factor that can induce the expression of certain EMT regulators and coordinate the interaction between these EMT regulators (such as E-cadherin and Vimentin) (29-31). The extracellular matrix serves an important role in the development of esophageal cancer (32). It has been shown that certain molecules are involved in these events, especially different collagen isoforms and enzymes associated with their metabolism, such as MMPs (32). Different MMP isoforms, especially MMP-2, -3, -7 and -9, have been reported to be involved in the development of esophageal cancer (32). The invasion and metastasis of esophageal cancer cells involve complex, continuous multi-step processes. Previous studies have revealed that there is a close association between MMP2 and the invasion and metastasis of esophageal cancer cells (33-35). High

MMP2 expression was observed in esophageal cancer tissue samples in the present study. Additionally, it has been found that MCU promotes the activity of MMP2 and cell movement, thereby promoting invasion, migration and metastasis of liver cancer cells (8).

Metastasis is one of the most important causes of death among patients with esophageal cancer and one of the important malignant phenotypes of cancer (13). Patients with esophageal cancer are prone to metastases to the lungs, brain and kidneys (36). The ability of cancer cells to invade and migrate is the basis of cancer metastasis. Strong invasive and migratory abilities help to break through the basement membrane and enter the lymphatic system or blood vessels, which eventually lead to the metastasis to lymph nodes or distant organs (13). The present results suggested that MCU accelerated proliferation and migration of esophageal cancer cells.

However, there were several limitations in the current study. Firstly, this was a retrospective study conducted at a single institution, with a relatively small sample size. The present results need to be further confirmed based on larger sample multi-center analysis. Secondly, the exact mechanism of esophageal cancer metastasis caused by MCU dysregulation is unclear and requires further study. In conclusion, the current data indicated that MCU was frequently expressed in esophageal cancer. Further research is required to confirm the application of MCU in the diagnosis and treatment of esophageal cancer.

Overall, the present study described for the first time the abnormal expression levels of MCU and its potential clinical value in esophageal cancer, providing a new potential biomarker and therapeutic target for esophageal cancer. However, the role of MCU in esophageal cancer requires further study.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

FZ conceived and designed the study. YM, XW, YL and WL conducted most of the experiments and data analysis, and wrote the manuscript. YH, HY and RH conducted immunohistochemistry and immunofluorescence experiments and data analysis and wrote and revised the manuscript. FZ and

YM confirmed the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of General Hospital of Ningxia Medical University (Yinchuan, China; approval no. 2016049). All patients provided written informed consent.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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