Upregulation of extracellular matrix metalloproteinase inducer promotes hypoxia-induced epithelial-mesenchymal transition in esophageal cancer

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Received September 29, 2014; Accepted July 17, 2015

DOI: 10.3892/mmr.2015.4410

Abstract. Extracellular matrix metalloproteinase inducer (EMMPRIN) exerts important roles in tumor progression, including angiogenesis, metastasis and therapy resistance. The epithelial-mesenchymal transition (EMT), which is induced by hypoxia, is an important process in cancer metastasis. However, the association between hypoxia and EMMPRIN remains to be elucidated in esophageal cancer. The expression of EMMPRIN was determined by western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and EMT markers were analyzed by western blotting, RT-qPCR and immunofluoresence. The migration and invasion of cells was investigated by Transwell assay. The results indicated that the expression levels of EMMPRIN in esophageal cancer cells were markedly higher compared with those in normal esophageal cells. EMMPRIN was able to promote esophageal cancer cell migration and invasion under both hypoxic or normoxic conditions, as demonstrated by the migration and invasion assay. The expression levels of E-cadherin were reduced, and those of snail family zinc finger 1, fibronectin, α-smooth muscle actin and fibroblast secretory protein 1 increased in esophageal cancer cells following treatment with human recombinant EMMPRIN under hypoxic conditions. The mRNA expression levels of the EMT markers were similar to those of the protein expression levels. Furthermore, the results demonstrated that EMMPRIN was regulated by hypoxia-inducible factor (HIF)-1α. These data suggested that EMMPRIN promoted metastasis and the EMT in esophageal cancer cells by regulating HIF-1α.

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Key words: esophageal cancer, epithelial-mesenchymal transition, metastasis, hypoxia

Introduction

Extracellular matrix metalloproteinase inducer (EMMPRIN) is a markedly glycosylated transmembrane glycoprotein (1-4). EMMPRIN is widely expressed in human tissues and exerts important roles in numerous tissue types, including the lung, thymus, retina, skin, cornea and nervous system, which involves various cellular processes (5,6). In oncology research, EMMPRIN has been the subject of numerous previous studies in the field of oncology, due to its consistently high expression levels on the surface of various tumor types (7-9). EMMPRIN is associated with cancer progression and exerts important functions in tumor migration, invasion, proliferation, angiogenesis, glycolysis and therapy resistance (7-9). EMMPRIN stimulates surrounding fibroblasts and endothelial cells to produce matrix metalloproteinases and urokinase in a paracrine fashion, leading to tumor cell invasion (10,11). Numerous previous reports demonstrated that the expression levels of EMMPRIN were significantly increased in esophageal cancer, as compared with adjacent tissues (12-20). This indicated that EMMPRIN may be a prognostic indicator for malignant tumors.

Hypoxia is a common characteristic of solid tumors, and promotes cancer cell proliferation, angiogenesis, apoptosis resistance, drug resistance and metastasis (21). Under hypoxic conditions, hypoxia-inducible factors (HIFs) are upregulated and affect various cellular biological processes, which allow the cancerous cells to adapt to their environments (22). HIF-1α is the most important subunit, and combines with a β subunit to form a heterodimer, which in turn has important roles in tumor hypoxia (22,23). Previous studies indicated that the expression of EMMPRIN is upregulated under ischemic conditions in neuronal and cardiac cells (24,25). A previous study demonstrated that the expression of EMMPRIN may be induced under hypoxic conditions in a colon carcinoma cell line, LS174 (26), and suggested the existence of an HIF-1 binding site, determined by chromatin immunoprecipitation-on-chip assay (27-29). However, the mechanism underlying the hypoxia-induced increase in the expression of EMMPRIN remains to be elucidated in esophageal cancer.

Based on these previous reports, the present study hypothesized that EMMPRIN has an important role in

HIF-1α-regulated metastasis and the epithelial-mesenchymal transition (EMT) in esophageal cells. The present study, therefore, investigated the expression of EMMPRIN in hypoxic esophageal cancer cells, as well as the role of EMMPRIN in metastasis and the EMT under hypoxic conditions.

Materials and methods

Cell culture and hypoxia treatment. Human esophageal cancer cell lines, including EC9709, EC-1 and EC-109, and the SHEE and HEEC normal esophageal cell lines were purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Australia) in a humidified incubator at 37˚C, containing 5% CO_2. For hypoxic exposure, the cells were placed in a HERAcell 240 hypoxia incubator (Thermo Fisher Scientific, Waltham, MA, USA) flushed with 1% O_2, 5% CO_2, and 94% N_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The total RNA (2 μg) was reverse transcribed using a SYBR® PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). To quantify the mRNA expression levels, qPCR was performed on a ABI Prism SDS 7000 sequence detector (Applied Biosystems Life Technologies, Foster City, CA, USA). The program of qPCR was as follows: 50°C 2 min, one cycle; 95°C 10 min, one cycle; 95°C 15 sec, 60°C 30 sec, 72°C 30 sec, 40 cycles; 72°C 10 min, one cycle. The relative expression levels were normalized to the levels of GAPDH and were analyzed using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). EMMPRIN and GAPDH primers were synthesized and obtained from Invitrogen Life Technologies. The primer sequences were as follows: EMMPRIN, forward 5'-CGGGGCTGCCGGCACAGTCTTC-3' and reverse 5'-AGCAGCCTCAGGTGGAACT-3'; and GAPDH, forward 5'-CATGACAACTTTGGTATCGTGG-3' and reverse 5'-CCTGCTTCACCACCTTCTTG-3'. The mRNA expression levels of EMMPRIN were normalized against those of GAPDH.

Small interfering (si)RNA transfection. HIF-1α-specific siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) was used to downregulate the expression of HIF-1α. A total of $2x10^5$ cancer cells were seeded into 6-well plates 24 h prior to transfection. The cells were transfected with HIF-1α siRNA or control siRNAs (100 nM) using Lipofectamine® 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions, and the medium was changed 6 h post-transfection. After 48 h, the total RNA or protein was extracted for RT-qPCR or western blotting, respectively.

Western blot analysis. Esophageal cancer cells were treated with human recombinant (hr)EMMPRIN (ACROBiosystems, Newark, DE, USA) or transfected with HIF-1α siRNA, and the total protein was isolated for western blotting. Briefly, the cells were lysed in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) to extract the proteins and the protein concentration was determined using a Bradford assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein was separated by 12.5% SDS-PAGE and transferred onto nitrocellulose (NC) membranes (EMD Millipore, Billerica, MA, USA) at 55 V for 4 h at 4˚C. The NC membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) and incubated with primary antibodies at 1:1,000 dilution in TBS overnight at 4˚C. The primary antibodies included mouse anti human EMMPRIN antibody (F-5; cat. no. sc-374101; 1:500), rabbit anti human E-cadherin antibody (H-108; cat. no. sc-7870; 1:500), mouse anti human fibronectin antibody (A-11; cat. no. sc-271098; 1:500), rabbit anti human SNAI 1 antibody (H-130; cat. no. sc-28199; 1:500), mouse anti human GAPDH antibody (G-9; cat. no. sc-365062; 1:500) and mouse anti human HIF-1α antibody (28b; cat. no. sc-13515; 1:500), which were purchased from Santa Cruz Biotechnology, Inc. Anti-αSMA (1A4; 1:2,000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-human FSP-1 antibody (cat. no. 13018; 1:1,000) was purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). The membranes were then washed three times (10 min each) in TBS, containing Tween 20 (TBST), and were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies, including goat-anti-mouse-horseradish-peroxidase (HRP)-conjugated IgG (cat. no. sc-2005; 1:5,000) and goat anti-rabbit HRP-conjugated IgG (cat. no. sc-2004; 1:5,000) from Santa Cruz Biotechnology, Inc., in TBST for 1 h at room temperature. The membranes were washed as before. The protein bands were visualized on X-ray film using an ECL Western Blotting kit (Pierce Biotechnology, Appleton, WI, USA). Protein band density was quantified using the gel-pro analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

Migration and invasion assay. For the migration assay, $1x10^5$ EC109 cells were plated into the top chamber on the non-coated membranes of 24-well plates (Corning Life Sciences, Lowell, MA, USA) and allowed to migrate toward the hrEMMPRIN-containing medium in the lower chamber. For the invasion assay, $1x10^5$ cells were plated into the top chamber of 24-well plates onto Matrigel coated membranes (BD Biosciences, Franklin Lakes, NJ, USA) and cultured for 24 h at 37˚C. Each insert was coated with 60 μg Matrigel prior to the invasion assay. The cells were added to medium with or without hrEMMPRIN. The cells that failed to invade through the pores were removed using a cotton swab. The invading cells were fixed with 10% methanol and subsequently stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The stained cells were counted under a microscope (BX51; Olympus Corporation, Toyko, Japan) at magnification, x40 in three random fields per well.

Immunofluorescence. The cells grown on sterile glass coverslips were briefly washed with PBS, fixed with cold 100% methanol for 5 min, and then air dried. The slides were first incubated with 10% species-specific serum for blocking for 30 min, followed by incubation with primary antibodies or nonspecific isotype antibodies (E-cadherin mouse monoclonal antibody; cat. no. sc-21791; Santa Cruz Biotechnology, Inc.)

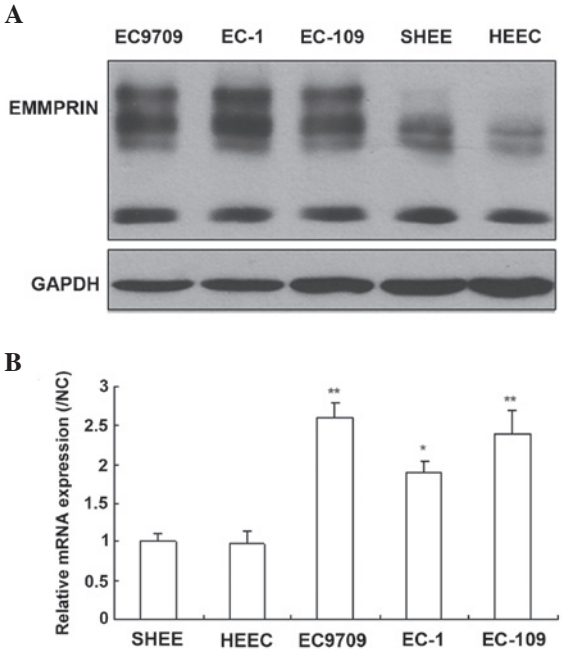

Figure 1. EMMPRIN is expressed in esophageal carcinoma cells. The cells were seeded into 6 cm culture plates prior to the experiment. The protein and mRNA expression levels were quantified by western blotting and reverse transcription-quantitative polymerase chain reaction, respectively. The (A) protein and (B) mRNA expression of EMMPRIN was markedly higher in the cancerous cell lines compared with the non-cancerous cell lines. *P<0.05 and **P<0.01, vs. control. EMMPRIN, extracellular matrix metalloproteinase inducer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

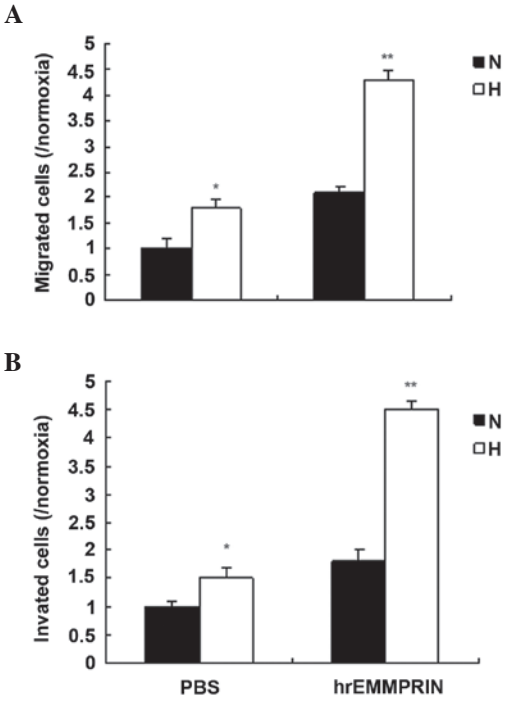

Figure 2. Treatment with EMMPRIN and hypoxia promotes metastasis. The cells were treated with hrEMMPRIN (10 μg/ml), and the migration and invasion ability was determined using transwell chambers. The EC-109 cells were plated in the upper chamber of the Transwell, and the medium in low chamber contained hrEMMPRIN or the control phosphate-buffered saline. The (A) migration and (B) invasion of the cells was increased in response to hypoxia and hrEMMPRIN treatment. *P<0.05 and **P<0.01, vs. control. N, normoxia; H, hypoxia; hrEMMPRIN, human recombinant extracellular matrix metalloproteinase inducer.

on ice overnight. Following incubation with the primary antibodies, the slides were washed twice with PBS, and were then incubated with FITC-labeled goat-anti-mouse IgG (1:300; cat. no. A11001; Invitrogen Life Technologies) in a dark chamber for 1 h at room temperature. The cell nuclei were stained with DAPI (1:20,000 in PBS). The slides were viewed under a microscope (BX51) and images were captured.

Statistical analysis. Each experiment was repeated in triplicate. The data are presented as the mean ± standard deviation. The statistical differences between the groups were analyzed by one or two-way analysis of variance, followed by Bonferoni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EMMPRIN is expressed in esophageal carcinoma cells. To investigate the role of EMMPRIN in esophageal carcinoma cells, the expression levels of EMMPRIN was quantified in both normal and cancerous esophageal cells by western blotting and RT-qPCR. The results demonstrated that the EMMPRIN protein was expressed in the majority of esophageal cancer cells, however, only marginally detectable in normal esophageal cells (Fig. 1A). The results of the RT-qPCR revealed that the mRNA expression levels of EMMPRIN were similar to the protein expression levels of EMMPRIN (Fig. 1B). These results suggested that EMMPRIN may exert an important function in the progression of esophageal carcinoma.

EMMPRIN promotes metastasis in a hypoxic environment. EMMPRIN has important roles in cancer metastasis, and a hypoxic environment is a common characteristic of solid tumors. To investigate the role of EMMPRIN in esophageal cancer, the migration and invasion ability of EC109 cells treated with hrEMMPRIN was analyzed using a Transwell assay. The results demonstrated that cell migration increased in the EC109 cells treated with hrEMMPRIN, and more cell migration and invasion were observed in hypoxic conditions, compared with the normoxic conditions (Fig. 2A and B).

EMMPRIN induces the EMT in hypoxic cells. The EMT is closely associated with metastasis. To investigate whether EMMPRIN is associated with the EMT, EMT markers, including E-cadherin, fibronectin, vimentin and α-smooth muscle actin (SMA) were detected by immunofluoresence and western blot analysis. The protein expression levels of the epithelial marker, E-cadherin, were markedly decreased, whereas those of fibronectin, vimentin , FSP1, Snail1 and α-SMA were markedly increased in the EC109 cells (Fig. 3A and 3B). In addition, the mRNA expression levels of E-cadherin were markedly decreased, whereas those of fibronectin, α-SMA, snail family zinc finger 1, and fibroblast secretory protein 1 were markedly increased in the EC109 cells, as determined by RT-qPCR (Fig. 3C).

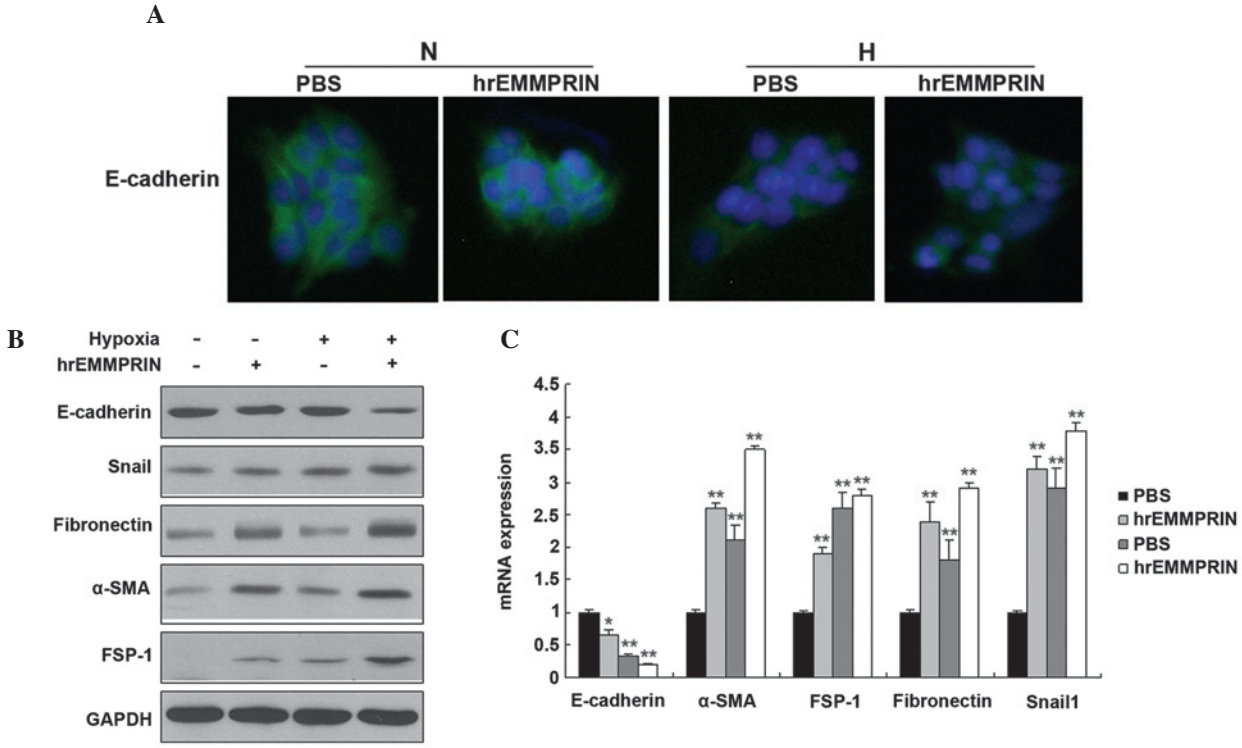

Figure 3. EMMPRIN induces the EMT when the cells are under hypoxic, however, not normoxic conditions. (A) The expression of E-cadherin was quantified by immunofluorescence. The EC109 cells were seeded into 12-well plates, treated with hypoxia (1% O_2) and hrEMMPRIN (10 μg/ml), fixed and incubated with E-cadherin and α-SMA antibodies. The cells were then analyzed using immunofluorescence microscopy. Green staining indicates the E-cadherin protein. (B) The expression levels of the EMT markers in pancreatic cancer cells were analyzed. The EC109 cells were seeded into 6-well plates, and treated with hypoxia (1% O_2) and hrEMMPRIN (10 μg/ml). The total protein was isolated from the cells and the protein expression levels of E-cadherin, fibronectin, α-SMA, FSP-1 and Snail1 were quantified by western blot analysis. The protein expression levels of Snail1, fibronectin, α-SMA and FSP-1 were increased, where as thh expression of E-cadherin was decreased. (C) The mRNA expression levels of E-cadherin, fibronectin, vimentin, α-SMA, FSP-1 and Snail1 were quantified by reverse transcription-quantitative polymerase chain reaction and revealed similar results to the protein expression levels. *P<0.05 and **P<0.01, vs. control. hrEMMPRIN, extracellular matrix metalloproteinase inducer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; α-SMA, α smooth muscle actin; FSP-1, fibroblast secretory protein 1; Snail1, snail family zinc finger 1; PBS, phosphate-buffered saline.

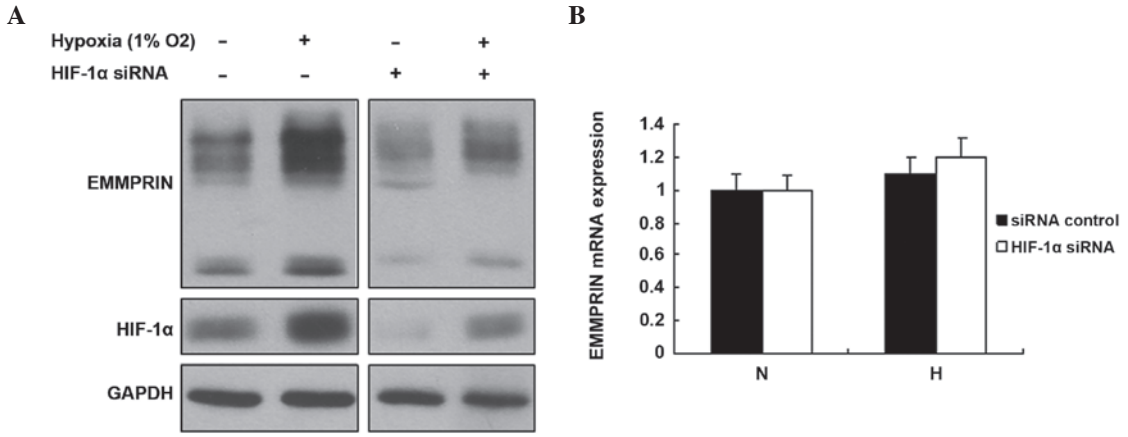

Figure 4. HIF-1α increases the expression levels of EMMPRIN in esophageal cancer cells. The EC109 cells were cultured under normoxia or hypoxia (1% O_2) for 24 h prior to siRNA transfection. (A) The total protein was isolated for western blotting in order to determine the protein expression levels of EMMPRIN and HIF-1α. (B) The total RNA was isolated for reverse transcription-quantitative polymerase chain reaction in order to determine the mRNA expression levels of EMMPRIN and HIF-1α. HIF-1α, hypoxia-inducible factor 1α; EMMPRIN, extracellular matrix metalloproteinase inducer; siRNA, small interfering RNA; N, normoxia; H, hypoxia.

HIF-1α increases the expression of EMMPRIN in esophageal cancer cells. HIF-1α is an important transcription factor, which regulates numerous target genes involved in various biological processes. Hypoxia increases esophageal cancer cell metastasis. In order to determine whether EMMPRIN was regulated by HIF-1α, the EC109 cells were treated with HIF-1α siRNA. The expression levels of EMMPRIN increased following hypoxic treatment, and conversely decreased following treatment with HIF-1α siRNA (Fig. 4A). No changes in the mRNA expression levels of EMMPRIN

were observed in the cells with downregulated HIF-1α (Fig. 4B). These data suggested that HIF-1α regulates the protein expression of EMMPRIN in esophageal cancer cells in a hypoxic microenvironment.

Discussion

Previous studies demonstrated that the expression of EMMPRIN was higher in esophageal cancer tissue samples, as compared with their normal counterparts (16,17) and that the expression of EMMPRIN was associated with lymph node metastasis, the severity of tumor invasion and differentiation (18-20). The present study demonstrated that the expression of EMMPRIN was higher in esophageal cancer cells compared with normal esophageal epithelial cells. However, the role of EMMPRIN in esophageal cancer remains to be elucidated. To the best of our knowledge, the present study provides the first evidence that EMMPRIN promotes the EMT of esophageal cancer in a hypoxic environment, and that EMMPRIN is regulated by HIF-1α.

The biological functions of upregulated expression of EMMPRIN were investigated under hypoxic conditions. Previous studies demonstrated that EMMPRIN increased the metastasis ability of cancer cells. In addition, previous reports demonstrated that EMMPRIN accelerated tumor cell invasion by stimulating the secretion of matrix metalloproteinase (7,8). The present study also demonstrated that the number of invading cells decreased following EMMPRIN suppression under hypoxic conditions. These results suggested that EMMPRIN may have an important role in hypoxia adaptation, promoting tumor cell survival and invasion. The analysis of cellular metastasis levels demonstrated that EMMPRIN increased esophageal cell migration and invasion under normoxic conditions, and this increase was further apparent under hypoxic conditions.

EMMPRIN promoted the EMT of cancer cells, including breast and colon cancer cells (8). However, in the present study, EMMPRIN revealed no induction of the EMT in esophageal cells under normoxic conditions, as determined by the absence of change in the expression levels of the EMT markers. Notably, under hypoxic conditions, EMMPRIN markedly increased the EMT in esophageal cells, and the expression levels of the epithelial marker, E-cadherin, decreased, whereas those of mesenchymal markers increased. These results indicated that EMMPRIN, combined with hypoxic conditions, induced the EMT.

Bioinformatic analysis determined the existence of eight putative hormone response elements (HREs) in the promoter region of EMMPRIN (19). HIF-1 directly binds to a specific HRE located at position 130-133 on the EMMPRIN promoter, and this may be involved in hypoxia-induced transactivation of EMMPRIN (28,29). The present study demonstrated that the expression of EMMPRIN was markedly upregulated by HIF-1α, which is a transcription factor with an important role in tumorigenic processes, including metastasis and the EMT.

In conclusion, the present study demonstrated that EMMPRIN promoted esophageal cancer migration and the EMT under hypoxic conditions via the expression of HIF-1α. Further research is required in order to investigate the molecular mechanism underlying the regulation of the EMT in esophageal cancer cells, and the association between EMMPRIN overexpression and hypoxic conditions.

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