ESM1/VEGFα/ERK signaling axis augments cell proliferation and tumor angiogenesis in human cervical squamous cell carcinoma

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Abstract

Background

Aberrant expression of endothelial cell specific molecule 1 (ESM1) is frequent in the carcinogenesis of various neoplasms. However, the expression profile and prognostic value of ESM1 in CSCC remain ill-defined.

Methods

Human specimens were utilized to investigate the expression of ESM1 in normal cervical tissue, LSIL, HSIL, and CSCC samples by IHC and RT-qPCR assay. And, it was further validated and explored in CSCC based on GEO and TCGA datasets. Then, genomic enrichment analysis (GSEA) and in vitro experiments of human CSCC cell lines, including SiHa and ME-180, were applied to probe the potential molecular mechanisms of ESM1 in CSCC.

Results

In human samples, the ESM1 was hyper-expressed in CSCC, compared with the normal ones. Combined with TCGA and GEO, it further revealed that ESM1 was significantly overexpressed and related to dismal prognosis in CSCC patients. And, GSEA analysis showed that the tumor angiogenesis and the VEGFα signaling pathway were mostly enriched in CSCC patients with ESM1 high expression. Then, the in vitro experiment suggested that interference of ESM1 inhibited cell proliferation, migration, invasion, and enhanced apoptosis, resulting in the reduction of VEGFα expression and the phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2) in SiHa and ME-180 cells.

Conclusions

ESM1 is notably overexpressed in CSCC patients. Overexpression of ESM1 predicts an adverse prognosis of CSCC. Overexpressed ESM1 augments tumor angiogenesis and progression of CSCC via the VEGFα/ERK signaling pathway. Thus, ESM1 and related genes may serve as promising prognostic biomarkers or candidate therapeutic targets for CSCC patients.

Introduction

Cervical squamous cell carcinoma (CSCC) is a popular gynecological malignancy with high morbidity and mortality. In the global world, cervical cancer accounts for approximately 15% of cancer-related deaths in the female group[1, 2]. The most common pathological type of cervical cancer is invasive squamous cell carcinoma, namely CSCC, which accounts for 75%-80% of cervical cancer[3]. Most CSCC is caused by the persistent infection of high-risk human papilloma virus (HR-HPV); The application of the
HPV vaccine especially the 9-valent HPV vaccine) has brought great benefit to the prevention of cervical cancer[4], but the mortality of cervical cancer have not significantly decreased in recent years[5]. According to the study, cervical cancer is ranked as the fourth leading cause of cancer-related deaths in females, with an estimation of 604,000 new cases and 342,000 deaths worldwide in 2020[6]. While mounting literature shows that the incidence and progression of CSCC is a pathological process caused by multiple factors and various signaling pathways[6–8]. Over the past decade, the vast amount of research on the molecular and genetic abnormalities that contribute to CSCC carcinogenesis has substantially expanded, giving an improved insight into the molecular mechanisms of cervical cancer and helping to guide the design of new therapeutic agents. It has been shown that somatic mutations in TP53[9], PIK3CA [10], KRAS[11], PTEN[12, 13], and STK11[14], as well as some copy number alterations, are relevant to the pathogenesis of cervical cancer[15, 16]. The methylation status of EPHX2 and RMI2 was closely related to the prognosis of CSCC[17]. It remains necessary and urgent to shed light on new molecular mechanisms of cell proliferation, apoptosis, and invasion in CSCC to derive effective biosignatures or therapeutic targets for disease diagnosis and prevention.

Endothelial cell specific molecule 1 (ESM1), also called endocan, is a proteoglycan widely distributed in vascular endothelium[18]. Studies have pointed out that ESM1 is involved in cancer progression or metastasis, including cell migration, invasion, tumor angiogenesis, and other pathological processes [19], and may be a latent biomarker for a variety of carcinomas[20]. Aberrant expression of ESM1 was found in a variety of malignancies, including hepatocellular carcinoma[21], triple-negative breast cancer[22], head and neck squamous cell carcinoma[23], prostate cancer[24], etc. However, the expression, prognosis, biological function, and process of ESM1 in CSCC remained largely unidentified.

This study combined IHC assay of human samples, analysis of Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases, and in vitro experiments to reveal that ESM1 was overexpressed in CSCC, associated with an unfavorable prognosis and involved in cell proliferation and tumor angiogenesis, as well as related signaling pathways. The findings of this essay help to widen the insight into CSCC, thoroughly analyze the significance of ESM1 in the pathogenesis and progression of CSCC and yield useful clues for early-stage diagnosis, novel prognostic biomarkers, or antitumor therapeutic targets in CSCC.

**Materials And Methods**

Human CSCC specimens

Under institutional ethical guidelines, all formalin-fixed and paraffin-embedded (PPFE) samples were gathered and then diagnosed and graded by at least two pathologists for the assessment of pathological features. Lastly, 45 cases of normal cervical tissues (the corresponding tissues were derived from uterine leiomyoma or inflammatory samples), 38 cases of LSIL, 32 cases of HSIL, and 145 cases of CSCC samples from Feb 2020 to Feb 2022 in our institution were collected for this study. All clinical samples were collected by informed consent (IFC) from patients or family members and this study has been
supported and approved by the Ethics Committee of Taihe Hospital. Clinically relevant parameters of all selected patients were illustrated in Additional file 1: Table S1.

Immunohistochemistry analysis

Immunohistochemistry (IHC) assay of PPFE tissues, including normal cervical tissues, LSIL (low-grade squamous intraepithelial lesion), HSIL (high-grade squamous intraepithelial lesion), and CSCC samples was performed according to the manufacturer's protocol. Specifically, all 3 µm sections cut from PPFE were dewaxed with xylene and rehydrated with graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Sections were incubated with primary antibody (Additional file 2: Table S2) at 4°C overnight and with HRP-labeled secondary antibody for 0.5 h at 37°C, and hematoxylin staining was performed for 30 seconds at 37°C. Then, ESM1-positive expressing cells were counted under high-power field (200× and 400×) microscopy. Three experienced pathologists scanned all IHC staining results and scored them. The grading criteria for cytoplasmic staining of ESM1-positive cells were set as follows: 0 (absent), 1 (< 10%, weak staining), 2 (10%-50%, moderate staining), or 3 (≥ 50%, strong staining). Cases with scores of 2 and 3 were recorded as strongly positive, namely ESM1 high expression subgroup. Otherwise, it was set as the ESM1 low expression subgroup (score = 0 and 1).

Quantitative real-time PCR (RT-qPCR)

The relative expression of the ESM1 was identified by using RT-qPCR, according to the merchant's protocol. Total RNA was isolated from FFPE blocks by RNeasy FFPE Kit (QIAGEN, Germany). Total RNA of 1 µg was reversely transcribed into cDNAs using Revert-Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). ESM1 was amplified in a 20-µL volume that contained 2 µL of cDNAs, 1 µl of forward and reverse primers (10 nmol/L) and 10 µL of PowerUp SYBR Green Master Mix (Thermo Scientific, USA) for 38 cycles (95°C for 15 s, 57°C for 15 s, 72°C for 30 s) after an initial 120 second denaturation at 95°C in an ABI Prism 7500 analyzer (Applied Biosystems, USA). GAPDH was utilized as an endogenous reference gene. All reactions were run in triplicate. The relative ESM1 mRNA expression was assessed by using the 2^{−ΔΔCt} method. All primers were manufactured by Sangon Biotech (Shanghai, China) and the corresponding sequences were presented in Additional file 3: Table S3.

The identification of ESM1 expression via bioinformatics strategy

In combination with GTEx, CSCC data from TCGA containing 22 normal cervical tissues, 253 CSCC, and 53 CSCA tissues were gained for further validation. Then, the GEO dataset, including GSE27333 and GSE64217, was screened for analysis of CSCC. Details of the corresponding GEO database were shown in Additional file 4: Table S4.

Correlation of ESM1 expression with clinicopathological parameters in CSCC
Due to the insufficient follow-up information on our clinical samples, the correlation of $ESM1$ expression with clinicopathological signatures in CSCC was further explored based on the TCGA database. Specifically, a total of 253 CSCC patients were assigned into two subgroups, including 126 specimens with $ESM1$ high expression ($ESM1^{\text{high}}$) and 127 specimens with $ESM1$ low expression ($ESM1^{\text{low}}$) by the median cutoffs. The 'ggrisk' package of R software (version 4.0.3) was applied to cluster the expression groups[25]. The distribution of $ESM1$ expression in ages, stages, grades, survival status, and other clinical characteristics for CSCC was displayed by the Sanguini diagram via the 'ggalluval' package [26]. Then, univariate cox (uni-cox) and multivariate cox (multi-cox) regression analyses were performed to develop the Nomogram via 'rms' R package[27]. Several characteristics, including p-values, hazard ratio (HR), and 95% confidence interval (CI) were presented by 'forest plot' R packages[28].

Prognosis of ESM1 in CSCC

The prognostic analysis of CSCC with $ESM1^{\text{high}}$ and $ESM1^{\text{low}}$ was conducted, including overall survival (OS) and progression-free survival (PFS).

Gene-set enrichment analysis (GSEA)

The underlying biological mechanisms of ESM1 in TCGA-CSCC, including 31 patients with the $ESM1^{\text{low}}$ subgroup (Top 25%) and 30 patients with the $ESM1^{\text{high}}$ subgroup (Top 25%) were probed via the GSEA software 4.2.2 (Broad Institute, USA). The three predefined gene-sets, including 'h.all.v7.2.symbols.gmt', 'c2.cp.go.v7.2.symbols.gmt' and 'c2.cp.biocarta.v7.2.symbols.gmt', were analyzed. The main statistical results of GSEA were set as the normalized enrichment score (NES) with a significance threshold: |NES| > 1 and normalized p-value < 0.05. In addition, the protein-protein interaction (PPI) network was assayed based on the STRING database (version 11.5) to elicit the interactions between ESM1 and related proteins.

The in vitro assay of SiHa and ME-180 cell lines

The siRNA assay and Western blotting

Human CSCC cell lines, including SiHa and ME-180 cells, were transfected with 50 nmol of ESM1 siRNA (siESM1), VEGFα siRNA (siVEGFα), or negative control siRNA (siNC) in the special medium (CM0451, Procell, China) for 48 h and 24 h, respectively, according to the manufacturer's instructions. Then, total RNA was isolated by TRIzol reagent (Invitrogen, USA) and RT-qPCR was performed as above described. The relative expression of ESM1 and VEGFα was calculated in three replicates. Meanwhile, other samples under the same culture conditions were used to lyse the protein for western blotting.

Cell proliferation assay

A total of 100 µL ($1 \times 10^4$ cells) of human SiHa or ME-180 cells were inoculated in 96-well plates and transfected with 50 nmol of siESM1, siVEGFα or siNC, followed by the addition of 10 µL of CCK-8 solution
(Beyotime, China) in each well. After 72 h or 48 h of incubation, the absorbance (optical density, OD) was measured at 450 nm, representing the density of cells.

Cell migration assay

Cell migration was analyzed by wound-healing assay. Human SiHa and ME-180 cells were plated in 12-well plates in DMEM containing 10% fetal bovine serum (FBS) and transfected with 50 nmol of siESM1, siVEGFα or siNC for 48 h and 24 h, respectively. The floating cells were washed with PBS and then 4 ml of DMEM (10% FBS, 1% antibiotic-antimycin) was added. Scratch areas were drawn at time endpoints.

Cell invasion assay

Human SiHa and ME-180 cells transfected with siESM1, siVEGFα, or siNC were cultured in TranswellR cell culture chamber (Corning, USA) at 1×10^4 cells/well for 48 h and 24 h, respectively. After 24 h of culture, cells under the membrane were fixed with 4% PFA and stained with crystal violet. Cells in 5 random areas of each chamber were photographed and counted using Image J software.

Cell apoptosis analyses

After transfection of human SiHa and ME-180 cells with siESM1, siVEGFα or siNC for 48 h and 24 h, respectively, the cells were disposed of with Annexin V-FITC kit (Beyotime Biotechnology, China) and analyzed by flow cytometry (FACSCalibur, Bio-Rad, USA) for the analysis of apoptotic cells.

Statistical analysis

GraphPad Prism 8.0 (San Diego, CA) software and SPSS 25.0 (IBM SPSS Inc., Chicago, IL) were utilized for statistical analysis. Results were presented as mean ± standard deviation (Mean ± SD). Comparisons between the two groups were conducted by One-Way ANOVA or two-tailed Student's t-test. The Kruskal-Wallis test was employed to evaluate the correlation between ESM1 expression and clinical characteristics. Uni-cox and multi-cox regression assays were deployed to characterize the effect of ESM1 expression on survival time and other clinical characteristics. The log-rank test was applied for prognostic analysis. Pearson's correlation test was used to check the correlation between the two variables. The threshold for statistical significance was set at p < 0.05 (ns = non-significant, * p < 0.05, ** p < 0.01, *** p < 0.001).

Results

ESM1 is overtly hyper-expressed in CSCC and HSIL, compared with LSIL or normal cervical tissues

The IHC assays were performed to probe ESM1 expression in human specimens, including normal cervical tissue, LSIL, HSIL, and CSCC samples. It suggested that the expression of ESM1 protein was no or low in normal cervical tissues, low in squamous cells of LISH, and significantly high in HSIL and CSCC. In LSIL and HSIL, the expression of ESM1 protein was mainly localized in the cytoplasm. However, the
ESM1 protein was not only localized in the cytoplasm but also extensively expressed in the intercellular matrix in CSCC. Consistent with the previous studies, the p16 protein was over-expressed in HPV-positive (HPV+) tissues and not expressed in normal (HPV-) tissues (Fig. 1A). The analysis of IHC scores indicated that ESM1 was remarkably hyper-expressed in CSCC or HSIL, compared with LSIL or normal cervical tissues ($p < 0.01$, Fig. 1B). Meanwhile, the relative expression of $ESM1$ mRNA was significantly upregulated in CSCC compared to normal subjects ($p < 0.0001$, Fig. 1D). The receiver operator characteristic (ROC) curve analysis suggested that the abnormal expression of ESM1 protein was highly accurate in predicting or diagnosing the prognosis of CSCC patients ($AUC = 0.91$, $95\% CI = 0.87–0.96$; Fig. 1E). Then, all CSCC patients ($n = 145$) were assigned to two subgroups, including 99 samples with ESM1 low expression (score = 0 and 1) and 46 samples with ESM1 high expression (score = 2 and 3) samples. Thus, the high expression of ESM1 (cases with a score of 2 or 3) was observed in 32% (46/145) of the CSCC samples (Fig. 1C). Additionally, the correlation analysis between ESM1 expression and clinicopathological parameters in CSCC figured out that the expression of ESM1 was not correlated with age, pathologic stage, clinical grade, and new tumor event type ($p > 0.05$, Additional file 1: Table S1). Compared with ESM1$^{\text{low}}$, CSCC patients with ESM1$^{\text{high}}$ were significantly featured with shorter survival time ($p = 0.023$, Additional file 1: Table S1). It hinted that ESM1 has prognostic significance and high expression might be unfavorable to CSCC.

The expression of ESM1 in the validation cohort based on bioinformatics analysis

To verify the results of the IHC assay, a bioinformatic analysis was conducted. It showed that the mRNA expression of $ESM1$ was dramatically overexpressed in CSCC, compared with normal ones based on GSE27333 ($p < 0.01$, Fig. 2A), GSE64217 ($p < 0.01$, Fig. 2B) and TCGA data repository ($p < 0.001$, Fig. 2C). Additionally, $ESM1$ was not associated with the histologic grade ($p > 0.05$, Fig. 2D) and clinical stage ($p > 0.05$, Fig. 2E). Consistent with the results of Fig. 1E, ROC curve analysis indicated that $ESM1$ mRNA expression was also highly accurate in predicting or diagnosing the prognosis of CSCC ($AUC = 0.905$, CI = $0.833–0.977$; Fig. 2F).

Correlation analysis of ESM1 and clinicopathological variables of CSCC patients

To investigate the correlation between $ESM1$ expression and clinicopathological variables in CSCC, all TCGA-CSCC cases were categorized into two subgroups, including 127 samples with ESM1$^{\text{low}}$ and 126 samples with ESM1$^{\text{high}}$, according to the median cut-off (Fig. 3A). Sanguini diagram described the distribution of $ESM1$ expression in age, pTNM stage, grades, and survival status (Fig. 3B). Then, the correlation between $ESM1$ and related clinical factors, including age and pTNM stage, on the OS in CSCC patients was determined by the uni-cox and multi-cox regression analysis. In the uni-cox analysis, $ESM1$ expression and pTNM stage were shown to be intimately coupled with OS in CSCC patients (all $p < 0.05$, Fig. 3C). In the multi-cox analysis, $ESM1$ expression could be an independent prognostic factor for CSCC patients (all $p < 0.05$, Fig. 3D). The Nomogram was finally employed to assess survival at 1, 2, or 3 years in CSCC patients with ESM1$^{\text{high}}$ (Fig. 3E-F).
Overexpression of ESM1 predicts an unfavorable prognosis for CSCC patients

Based on prognostic analysis of the TCGA-CSCC dataset, overexpressed ESM1 indicates a poor OS (HR = 1.96, log-rank $p = 0.0117$; Fig. 3G,) and PFS (HR = 1.95, log-rank $p = 0.0146$; Fig. 3H) in CSCC.

GSEA reveals hidden molecular mechanisms of ESM1 in the initiation and progression of CSCC

The analysis of GSEA was used to profile cancer-related signaling pathways enriched in CSCC with ESM1 high expression. According to GSEA enrichment analysis of the Biocarta pathway, CSCC patients with ESM1$^{\text{high}}$ were mostly enrolled in the VEGF pathway (NES = 2.03, $p = 0$, Fig. 4A; Additional file 5: Table S5). The GSEA analysis of GO terms uncovered that the overexpression of ESM1 was tightly related to the positive regulation of endothelial cell proliferation (NES = 1.93, $p = 0$, Fig. 4A; Additional file 6: Table S6). Additionally, The GSEA analysis of the Hallmark description revealed angiogenesis (NES = 1.67, $p = 0.03$, Fig. 4A; Additional file 1: Table S7) and hypoxia (NES = 1.80, $p = 0.04$, Fig. 4A; Additional file 7: Table S7) were mostly involved in CSCC patients with ESM1$^{\text{high}}$. Then, Circos's graph suggested that VEGF$\alpha$ and HIF-1$\alpha$ were co-expressed and exerted a crucial role in these four pathways (Fig. 4B). Based on the STRING database, the PPI network displayed that close interactions were revealed among ESM1, VEGF$\alpha$, HIF-1$\alpha$, and VEGFR2/3 (Fig. 4C), which also has been reported in the existing literature [19, 29]. By further filtering the correlation and prognosis of VEGF$\alpha$ and HIF-1$\alpha$ in CSCC, it was noted that VEGF$\alpha$ and HIF-1$\alpha$ have a positive correlation with the expression of ESM1 (all $p < 0.001$, Fig. 4D). And, overexpressed VEGF$\alpha$ was related to the adverse OS of CSCC patients (HR = 1.75, $p = 0.033$, Fig. 4E), but not HIF-1$\alpha$ (HR = 1.62, $p = 0.069$, Fig. 4E). Further, the IHC revealed that VEGF$\alpha$ and HIF-1$\alpha$ were overexpressed in CSCC, compared with normal counterparts. Besides, the expression of CD31, a marker of vascular endothelium, was upregulated in CSCC (Fig. 4F). Thus, it suggested that ESM1 might play a vital role in regulating the tumor angiogenesis and progression of CSCC by interacting with VEGF$\alpha$ and HIF-1$\alpha$.

ESM1 interference affects cell proliferation, invasion, and apoptosis in SiHa and ME-180 cells

To further investigate the role of ESM1 in CSCC, in vitro experiments of ESM1 siRNAs (siESM1) were performed in human CSCC cell lines, including SiHa and ME-180 cells. The ESM1 expression was significantly inhibited in siESM1 compared to siNC in terms of mRNA and protein (Fig. 5A-B). In the cell proliferation assay, ESM1 interference significantly suppressed cell proliferation (Fig. 5E-F) and cell migration (Fig. 5G-H) in human SiHa and ME-180 cells after being transfected with siRNAs for 48 h and 24h, respectively. Inhibition of ESM1 significantly diminished cell invasion in human SiHa (Fig. 5I) and ME-180 (Fig. 5J) cells. Finally, flow cytometry assays indicated that down-regulation of ESM1 distinctly enhanced cell apoptosis in human SiHa (8.74% vs 27.2%, Fig. 5K) and ME-180 cells (19.3% vs 31.8%, Fig. 5L).

As analyzed in Fig. 4, the expression of ESM1 was closely correlated with VEGF$\alpha$ in CSCC. Thus, we designed siVEGF$\alpha$ to probe the correlation between ESM1 and VEGF$\alpha$ (Fig. 5C-D). Down-regulation of VEGF$\alpha$ markedly inhibited cell proliferation (Fig. 5E-F), cell mobility (Fig. 5G-H) and cell invasion (Fig. 5I-J) in human SiHa and ME-180 cells after transfected with siRNAs for 48 h and 24 h, respectively. Eventually,
Flow cytometry experiments showed that inhibition of VEGFα significantly enhanced cell apoptosis in human SiHa (8.74% vs 23.5%, Fig. 5K) and ME-180 cells (19.3% vs 33.9%, Fig. 5L).

ESM1 regulates HIF-1α expression and VEGFα signaling pathway in SiHa and ME-180 cells

Based on western blotting experiments, the molecular mechanisms of ESM1 were further discussed in CSCC cell lines, including SiHa and ME-180 cells. Suppression of ESM1 expression led to the decrease in VEGFα expression, HIF-1α expression, and phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2). Meanwhile, inhibition of VEGFα also significantly reduced the expression of ESM1, HIF-1α, and phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2) in SiHa (Fig. 5M) and ME-180 cells (Fig. 5N).

Discussion

Cervical cancer, mainly including CSCC, is characterized by a high degree of malignancy, a gradual increase in the incidence rate, and a younger incidence group[30, 31]. The main therapy for early-stage cervical cancer is surgical resection; Patients who have lost the opportunity of surgery in advanced stages require radiotherapy and chemotherapy, but the effect is still unsatisfactory[5]. For patients with cervical cancer who fail to receive the HPV vaccine within the defined age range or who are not HPV-related cervical cancer, it is of great value for screening and improving the clinical outcome or prognosis of cervical cancer to find early-stage diagnostic markers, prognosis indicators, or new therapeutic targets.

ESM1 is a 50 kDa soluble proteoglycan and is mainly secreted by vascular endothelial cells. In normal tissues, ESM1 is mainly expressed in vascular endothelial cells, distal renal tubular epithelial cells, and pulmonary endothelial cells, and is mainly involved in the inflammatory response[32] and angiogenesis[33]. Accumulative studies have shown that ESM1 was hyper-expressed in a variety of malignancies and was associated with tumor prognosis[34]. In hepatocellular carcinoma, ESM1 was an early-stage biomarker via augmenting the formation of tumor angiogenesis[21]. In TNBC (triple-negative breast cancer), ESM1 promoted the cell migration, proliferation, and invasion of TNBC cells by Akt-dependent NF-κ B/Cyclin D1 signaling pathway[22]. In esophageal carcinoma, the expression of ESM1 mRNA and protein was significantly up-regulated, which was associated with a poor prognosis. ESM1 was involved in the cell proliferation, migration, and Janus kinase (JAK) signaling pathway of esophageal cancer cells. Interference with ESM1 expression would significantly inhibit the proliferation and migration of esophageal cancer cells and reduce the JAK1 protein expression[20]. In prostate cancer, overexpressed ESM1 was associated with poor OS and advanced metastasis. ESM1 maintained the stemness and metastasis of prostate cancer cells directly by activating the Wnt/β-Catenin signaling pathway[24]. Additionally, ESM1 and the co-expressed gene, ANGPT2, were significantly overexpressed in head and neck squamous cell carcinoma (HNSC). ESM1 might promote the progression of HNSC through the Ras-MAPK-ERK signaling pathway[23]. In addition, as a glycoprotein secreted into the bloodstream, ESM1 mediated the progression of EGFR-driven non-small cell lung cancer[35]. Nevertheless, the expression alteration, molecular mechanisms, and biological functions of ESM1 in CSCC remain poorly
understood. The purpose of this essay is to systematically investigate the expression pattern, prognosis, and latent function of ESM1 in CSCC.

The IHC assay revealed that the expression of ESM1 was no or low in normal cervical tissues, low in squamous cells of LISH, and significantly high in HSIL and CSCC. However, the ESM1 protein was not only localized in the cytoplasm but also extensively expressed in the intercellular matrix in CSCC. It indicated that ESM1 might serve a crucial driving role during the process from LISH to CSCC. And, episomal secretion of ESM1 protein into the interstitium may play a key role in the development of CSCC. Additionally, the high expression of ESM1 (cases with an IHC score of 2 or 3) was observed in 32% (46/145) of the CSCC samples. Correlation analysis of ESM1 expression in CSCC with clinicopathological variables figured out that, the CSCC patients with ESM1\textsuperscript{high} were not correlated with age, pathologic stage, clinical grade, and new tumor event type, but were closely related to shorter survival time. It hinted that ESM1 had prognostic significance, and high expression might be unfavorable to CSCC patients. Coupled with the RT-qPCR assay, the ROC analysis of mRNA indicated that ESM1 harbored high accuracy in predicting or diagnosing the prognosis of CSCC patients. Then, a further understanding of ESM1 expression was achieved by examining CSCC-related datasets (GEO and TCGA) that reinforced the above results. In addition, the uni-cox and multi-cox analysis based on the TCGA data repository indicated that ESM1 expression was associated with OS in CSCC patients and might serve as an independent prognostic factor for CSCC patients. The prognosis of ESM1 was assessed and showed that overexpression of ESM1 was significantly associated with poor OS and PFS in CSCC patients. A recent study stated that overexpression of ESM1 might enhance cancer progression and metastasis by regulating tumor cell proliferation, migration, invasion, and drug-resistant, and was involved in the tumor microenvironment compassing inflammation and angiogenesis \cite{36}. Consequently, the GSEA suggested that ESM1 was mostly enriched in the VEGF\textalpha signaling pathway; ESM1 might play a pivotal role in regulating the tumor angiogenesis and progression of CSCC by interacting with VEGF\textalpha and HIF-1\alpha.

To validate the above GSEA results, we established a siRNA system to explore the latent biological functions and molecular mechanisms by conducting \textit{in vitro} experiments in human CSCC cell lines, including SiHa and ME-180 cells. It suggested that ESM1 interference inhibited cell proliferation, migration, and invasion and enhanced apoptosis of SiHa and ME-180 cells. Namely, overexpression of ESM1 induced cell proliferation, migration, and invasion and inhibited cell apoptosis. These results indicated that ESM1 might play a vital role in regulating cell proliferation, migration, invasion, and anti-apoptosis in CSCC. By western blotting, interference of ESM1 by siRNA resulted in the decrease of VEGF\textalpha expression, HIF-1\alpha expression, and the phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2). Hence, the hyper-expression of ESM1 upregulated the expression levels of VEGF\textalpha, and HIF-1\alpha, and activated VEGF\textalpha/VEGFR2/ERK signaling pathway via the phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2). By contrast, interference of VEGF\textalpha by siRNA resulted in the decrease of ESM1 expression, HIF-1\alpha expression, and the phosphorylation of VEGFR2 (P-VEGFR2) and ERK-1/2 (P-ERK-1/2). Combined with the PPI network, it suggested that ESM1 regulated HIF-1\alpha expression and VEGF\textalpha signaling pathway in SiHa and ME-180 cells.
Previous literature reports enhanced our results. The HIF-1α regulated the expression of VEGFα, thus resulting in angiogenesis and an unfavorable prognosis in cancer[37, 38]. Malignant tumor tissue not only had the characteristics of rapid proliferation but also formed highly hypoxic regions and specific tumor microvessels in the tissue[39, 40]. VEGFα played a key role in tumor angiogenesis; And, whereas HIF-1α played a central role in regulating the signal transduction pathway of VEGFα during hypoxia. It functioned not only to improve the mRNA stability of VEGFα but also to enhance the transcriptional activity of VEGFα. Inhibition of VEGFα/HIF-1α expression can inhibit tumor angiogenesis[41]. Additionally, Susana F et al. reported that the bioavailability of VEGFα was enhanced by ESM1 upregulation to modulate endothelial cell proliferation and vascular permeability based on the ESM1 knockout mice model[19]. In SiHa and ME-180 cells, interference of ESM1 resulted in the decrease of VEGFα and HIF-1α expression. Meanwhile, interference of VEGFα resulted in the decrease of ESM1 and HIF-1α expression. Combined with previous related research, it hinted that the overexpressed ESM1, VEGFα, and HIF-1α harbored synergistic effects in the process of tumorigenesis and progression of CSCC cells via the VEGFα/VEGFR2/ERK signaling pathway (Fig. 6).

In the present paper, we have addressed the potential biological function of hyper-expressed ESM1 in augmenting endothelial cells proliferation and tumor angiogenesis via the VEGFα/ERK signaling pathway in human CSCC, suggesting that ESM1 might function as a potential prognostic marker or therapeutic target for CSCC. Nonetheless, there are still some limitations in our study. Firstly, the prognosis analysis and potential molecular mechanisms of ESM1 in CSCC patients are mainly based on the analysis of the TCGA database; Secondly, this study lacks some of our own clinical information, such as tumor subtypes and follow-up data, which may put a limit on the value of the clinical application of ESM1 in CSCC. Then, in vitro experiments with siRNA cell lines have explored the biological functions of ESM1, but in vivo experiments or further studies of ESM1 in CSCC patients are still essential. Finally, as one of the secreted proteins, ESM1 expression in the bloodstream of CSCC patients has not been obtained, so it is necessary to further discuss the potential diagnostic or prognostic value of ESM1 in the blood sample of CSCC patients. Therefore, further in vivo experiments combined with blood samples from CSCC patients are still necessary to validate the above findings.

Conclusions

This essay showed that ESM1 was significantly overexpressed in CSCC patients. Overexpression of ESM1 heralded a dismal prognosis of CSCC and augmented the progression of CSCC. Thus, ESM1 could be used as a prognostic biomarker or therapeutic target for CSCC patients. According to the results of in vitro experiments, overexpressed ESM1, VEGFα, and HIF-1α harbored synergistic effects in the tumorigenesis and progression of CSCC cells through the VEGFα/VEGFR2/ERK signaling pathway. Briefly, this study revealed significant evidence of ESM1 and related signaling pathways, which might shed useful light on the development of individualized treatment strategies for CSCC patients with high ESM1 expression.
Abbreviations

CSCC
Cervical squamous cell carcinoma
ESM1
Endothelial cell-specific molecule 1
VEGFα
Vascular endothelial growth factorα
HPV
Human papilloma virus
CIN
Cervical intraepithelial neoplasia
HIF-1α
Hypoxia-inducible factor 1 alpha
RT-qPCR
Reverse transcription-quantitative PCR
IHC
Immunohistochemistry
CCK-8
Cell Counting Kit-8
TCGA
The Cancer Genome Atlas
GEO
Gene Expression Omnibus
OS
Overall survival
PFS
Progression-free survival
ROC
Receiver operator characteristic
HR
hazard ratio
CI
Confidence interval
GSEA
Gene set enrichment analysis
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
STRING
The Search Tool for the Retrieval of Interacting Genes
SD
Standard Deviation.

Declarations

Acknowledgments

Not applicable.

Author contributions

Yu-gang Huang, Dan Li, and Xian-bin Tang conceived, designed, and wrote the paper. Xiao-min Su conducted the in vitro assay and supervised the research; Yu-gang Huang and Li Yao performed the IHC assay and statistical analysis; Li Wang and Dan Yu were involved in the study design and offered helpful advice on methods and chart preparation. All authors have read and approved the final version of the manuscript.

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Availability of data and materials


Ethics approval and consent to participate

All human samples were obtained by informed consent (IFC) from patients or family members, and this study was supported and approved by the Ethics Committee of Taihe Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

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References


Figure 1

The IHC staining and RT-qPCR assay of ESM1 expression in CSCC and normal cervical tissues. (A) The expression of ESM1 and p16 in CSCC, HSIL, LSIL, and normal cervical tissues via IHC (high power fields, 200× or 400×). LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion. (B) The ESM1 score of IHC images in CSCC (n=145), HSIL (n=32), LSIL (n=38) and normal cervical tissues (n=45). (C) The percentage of including low expression group (score=0 and 1) and high expression group (score=2 and 3) in normal and CSCC samples. (D) The mRNA level of ESM1 in
normal cervical tissues (n=45) and CSCC (n=145) by RT-qPCR assay. (E) The ROC curve analysis based on *ESM1* mRNA expression in normal cervical tissues and CSCC. CI, confidence interval.

**Figure 2**

*ESM1* mRNA was overexpressed in CSCC patients, compared with normal control. The relative expression of *ESM1* mRNA from GSE27333 (A), GSE64217 (B), and TCGA-GTEx datasets (C). Relative expression of *ESM1* mRNA in histologic grade 1-4 (D) and clinical stage I-IV (E) based on TCGA dataset. (F) The ROC curve analysis of *ESM1* expression in TCGA-CSCC patients.
Figure 3

The ESM1 prognostic analysis in the clinicopathological variables of CSCC patients. (A) ESM1 expression (low or high) and survival status (dead or alive) in CSCC patients. The order of all samples was consistent. (B) Sanguini plot for the analysis of the distribution of ESM1 expression in age, pTNM stage, grade, and survival status. The uni-cox (C) and multi-cox (D) analysis of ESM1 and relevant characteristics. (E) Nomogram to assess 1-, 2- and 3-year survival rates in CSCC patients associated with
high ESM1 expression. (F) Calibration curves of the Nomogram model for the OS of CSCC patients. The diagonal dashed line denotes the ideal Nomogram, and the blue, red, and orange lines denote the 1-y, 2-y and 3-y observed Nomograms. The closer the Nomogram model is to the calibration curve, the better the model predicts the results. The prognosis of ESM1 expression in CSCC patients, including the OS (G) and PFS (H).
ESM1 regulates the tumor angiogenesis and progression of CSCC by interacting with VEGFα and HIF-1α. (A) GSEA enrichment analysis of the Biocarta pathway, GO terms, and Hallmark description mainly involved in CSCC with ESM1\textsuperscript{high}. (B) Circos's graph of GSEA enrichment analysis revealed the signaling pathways and related co-expressed genes. (C) PPI network of co-expressed genes. (D) Correlation analysis between VEGFα, HIF-1α, and ESM1 expression in CSCC. (E) OS of VEGFα and HIF-1α expression in CSCC based on TCGA database. (F) IHC assay of VEGFα, HIF-1α, and CD31 protein in CSCC and normal ones.
Figure 5

ESM1 is involved in cell proliferation, migration, and apoptosis of SiHa and ME-180 cells. ESM1 expression was measured by (A, C) RT-qPCR and (B, D) western blotting in human SiHa and ME-180 cells after being transfected with siESM1, siVEGFα, or siNC. (E, F) Cell proliferation experiment of SiHa or ME-180 cells transfected with siESM1, siVEGFα, or siNC for 72 h and 48 h, respectively. (G, H) Cell mobility assay of SiHa or ME-180 cells transfected with siESM1, siVEGFα, or siNC for 48 h and 24 h, respectively; (I, J) Cell invasion assay of SiHa or ME-180 cells transfected with siESM1, siVEGFα, or siNC for 48 h and 24 h, respectively. (K, L) Apoptosis assay of SiHa or ME-180 cells transfected with siESM1, siVEGFα, or siNC for 48 h and 24 h, respectively. (M, N) Western blotting for ESM1, VEGFα, P-VEGFR2, VEGFR2, P-ERK1/2, ERK1/2, and HIF-1α in human SiHa and ME-180 cells transfected with siESM1, siVEGFα or siNC for 48 h and 24 h, respectively.
Figure 6

Molecular mechanism diagram. The overexpression of ESM1 induces the expression of VEGFα and HIF-1α, activates the VEGFα/VEGFR2/ERK signaling pathway, boosts angiogenesis, tumor cell proliferation, and invasion, and inhibits apoptosis. Thus, ESM1 is an important factor in angiogenesis and cellular resistance to apoptosis during tumorigenesis and the progression of CSCC.
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