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Inhibition of *EFEMP2* Suppresses the Cell Proliferation of Cervical Cancer Cells *via* AKT Signaling

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Abstract

Objectives: Cervical cancer is the most common gynecological cancer in the world and is one of the leading causes of cancer related death among women. *EFEMP2*, an extracellular matrix protein that could be easily detected in serum, plays key roles in several cancers formation and progression. However, its role in cervical cancer remains to be determined.

Methods: We have analyzed differentially expressed genes between Long-Term Survivors (LTSs) and Short-Term Survivors (STSs) using edgeR. The *EFEMP2* shRNA plasmids were prepared and the *EFEMP2* knockdown cervical cancer cell lines were constructed. The functions of *EFEMP2* on cervical cancer were evaluated *in vitro* and *in vivo* and the molecular mechanism was investigated by western blotting, etc.

Results: The genes associated with the Extracellular Matrix (ECM) were significantly down-regulated in LTSs group compared with STSs. Low expression of *EFEMP2* was positively associated with longer survival of cervical cancer patients. Knockdown of *EFEMP2* not only suppressed the cell proliferation of cervical cancer cells *in vitro,* but inhibited the tumor growth *in vivo*. Moreover, *EFEMP2* knockdown suppressed the expression of several genes related to promoting-proliferation by inhibiting AKT signaling.

Conclusion: *EFEMP2* functions as an oncogene to promote cell proliferation of cervical cancer, and it is a promising biomarker and therapy target for human cervical cancer.

Keywords: Extracellular Matrix; *EFEMP2*; Cervical Cancer; Tumor Biomarkers; AKT Signaling

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Introduction

Cervical cancer is the second most common cancer among global females, which ranks next only to breast cancer and becomes one of the main death causes for females [1]. 527,600 new cases were added and the death toll reaches 265,700 in 2012 [1]. It causes serious harm on developing countries particularly [1]. For example, cervical cancer is the main reason to cause female death in Africa, Central America and Northeast Asia [2]. Cervical cancer is the gynecological oncology with the highest morbidity and mortality in China [3]. There were 98,900 new cases, wherein 30,500 cases were died in China in 2015 [3]. Although the cervical cancer screening has been applied for decades, 5-year survival rate of cervical cancer is not improved remarkably [1]. Actually, the research on cervical cancer has been developed early, since the first immortalized human cell line-Hela is taken from a black woman with cervical cancer [4]. However, the pathogenesis of cervical cancer and its molecular mechanism remains to be determined. It is of great significance to disclose the molecular mechanism for tumor diagnosis and treatment. For example, cell cycle regulating protein CDK4 promotes tumorigenesis and growth [5]. Palbociclib, a newly-approved selected CDK4 inhibitor, has remarkable treatment effect on advanced breast cancer [6]. Therefore, it is very necessary to study a novel and effective biomarker or therapy target related with tumorigenesis and progression of cervical cancer.

The Extracellular Matrix (ECM) is a highly dynamic fundamental structure that is present in all tissues [7-9]. The ECM serves not only as the scaffold upon which tissues are organized but also provides critical biochemical and biomechanical signals that regulate cell growth, survival, migration, differentiation and immune function [8,10,11]. Similarly, besides functioning as an integral feature, the ECM actively contributes to the formation and progression of cancer [12,13].

For instance, pancreatic cancer patients usually show a marked stromal desmoplasia which associates with rapid progression and poor prognosis [14]. Similarly, MMPs, the matrix remodeling genes, are predictive of a poor prognosis for breast cancer patients [15]. Laminins, one of the main components of ECM, impact the migration and functions of immune cells [16]. Although the ECM has explained many features of tumor, there are many unanswered questions with regard to how the ECM and its biophysical properties influence tumor progression.

Fibrous is an ECM glycoprotein, consisting of epidermal growth factor EGF-like domain, calcium combined with EGF-like domain and unique fibrin C terminal domain [17]. The fibrous not only stabilizes the ECM structure, but also interacts with cells and control the growth of normal and malignant cells [18]. For example, Yasmin et al. [19] reported that Fibulin1 plays an extremely important role in determining aortic stiffness and its modification with age could underpin aortic stiffening. Fibulin-2 is critically associated with breast cancer progression [20]. EGF-containing fibulin-like extracellular matrix protein 2 (*EFEMP2*, called as Fibulin-4), a fibulin member, is proved to have an indispensable role in the formation of collagenous fiber and elastic fiber [21]. It is reported that the mice with knockout *EFEMP2* lack elastic fiber and are dead during the perinatal period [22]. It is illustrated that *EFEMP2* is markedly increased in colorectal cancer tissue and could function as serum biomarker for the early detection of colorectal cancer [23]. Additionally, the high expression of *EFEMP2* can remarkably promote the proliferation and invasion of glioma [24]. Although the functions of *EFEMP2* have been investigated in several human cancers, its role involved in cervical cancer remains to be further determined.

Materials and Methods

Cervical squamous cell carcinoma data in TCGA

Clinical data and RNA-seq data of primary CESC samples in The Cancer Genome Atlas (TCGA) were downloaded from Genome Data Commons (https://portal.gdc.cancer.gov). Long-Term Survivors (LTSs) were defined as patients with overall survival of >5 years from surgery, Short Term Survivors (STSs) as patients with survival >30 days and <5 year from surgery, to exclude patients of perioperative mortalities.

Differentially expressed genes analysis and Gene Set Enrichment Analysis (GSEA)

Differentially expressed genes analysis between 37 LTSs and 213 STSs were analyzed by edgeR R/Bioconductor package [25]. GSEA was performed *via* GSEA Pre ranked tool in GSEA software (http://www.broadinstitute.org/gsea/) [26]. All genes and their corresponding log2 (FC) values calculated by edgeR package were used as input data. We used the curated canonical pathways sub-collection of C2 collection in the Molecular Signatures Database (MSigDB) as the gene sets in our analyses.

Cell culture

The human cervical cancer cell line Hela and Siha, as well as the 293T cell line, were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Hela, Siha and 293T cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Hyclone, Logan, Utah) at 37°C and 5% CO_2.

Plasmids transfection and lentivirus infection

The shRNAs target human *EFEMP2* sequences were obtained from Sigma-Aldrich (Table 1). For viral packaging, 293T cells were transfected with pLVX-shRNA2, psPAX2 and pMD2.G plasmids using the FuGene HD transfection reagent (Roche Diagnostics, Basel, Switzerland). Forty eight hours after transfection, the virus-containing medium was harvested and filtered to remove cell debris. The cervical cancer cells were infected with virus-containing medium for 24 h then replaced fresh medium. The infected cells displayed green fluorescence. Five days after infection, the stably infected clones were selected with Flow Cytometer (BD FACS AriaII, BD Biosciences, Franklin Lakes, NJ).The selected stably infected clones were incubated for further study.

Cell proliferation assay

For the proliferation assay, Hela and Siha cells were seeded into 96-well plates (1000 cells/well) and were detected using CCK8 at 12 h, 36 h, 60 h and 84 h. In brief, 10 μl cell counting Kit-8 reagent (CCK8) (Dojindo, Kumamoto, Japan) was added into each well of the 96-well plate. Then the plate was incubated at 37°C for 2 h. The absorbance at 450 nm was subsequently detected with SpectraMax M5 (Molecular Devices, Silicon Valley, CA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed to detect the transcriptional level of *EFEMP2* according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, total cellular RNA was extracted from Hela and Siha cells with TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc.). Then the TRIzol mixed with chloroform (in a 5:1 ratio) followed by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant was blended with isopropanol (in a 1:1 ratio) for 30 min at room temperature and then centrifuged at 10,000 rpm for 10 min at 4°C. The RNA precipitate was washed with 75% ethanol and solubilized with Diethypyrocarbonate (DEPC) treated water. The RNA quantity and quality was measured at 260 nm/280 nm using the NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). Single stranded cDNA was prepared using a Reverse Transcription System (Baosheng, Dalian, China) following the operation manual. mRNA level of *EFEMP2* was determined with a SYBR Green qPCR Mix kit (Baosheng). qPCR was performed in an Applied Biosystems 7300 sequence detection system (Applied Biosystems, Thermo Fisher Scientific). All experiments were performed in triplicate. The expression levels of relative genes were calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in this study were presented in Table 1.

Western blotting

The expression of genes in Hela and Siha cells from different treatment groups were detected by western blotting. After being washed twice with ice-cold PBS, the cells were lysed in 2X SDS sample buffer (10 mM EDTA, 4% SDS, 10% glycine in 100 mMTris-HCl buffer, pH 6.8) for 1 hour at 4°C. The cellular lysates were then centrifuged (12,000 rpm for 15 min at 4°C), and the protein concentration was detected using the BCA protein assay kit (Beyondtime, Nantong, China). Cell lysates (20 μg of total proteins) were separated by 10% SDS-PAGE and electro-blotted onto a PVDF membrane (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 3% BSA for 1 h at room temperature, and then incubated with different primary antibodies (1:1000): anti-*EFEMP2* (#ab125073; Abcam, Cambridge, UK), anti-CYCLIN E1 antibody (#ab33911; Abcam), anti-p21 (#ab134175; Abcam), anti-AKT1 (#ab227100;

Table: The sequences of qRT-PCR and sh *EFEMP2* used in this study.

Notes	Name	Sequences
qRT-PCR	*EFEMP2* F	AAGAGCCCGACAGCTACAC
	EFEMP2 R	AGGGATGGTCAGACACTCGTT
***EFEMP2* shRNA**	*EFEMP2*	CCGGCCCTGATGGTTACCGCAAGATCTCGAGATCTTGCG
	shRNA 1F	GTAACCATCAGGGTTTTTG
	EFEMP2	AATTCAAAAACCCTGATGGTTACCGCAAGATCTCGAGAT
	shRNA 1R	CTTGCGGTAACCATCAGGG
	EFEMP2	CCGGGCTTCTCCTGCAGTGATATTGCTCGAGCAATATCA
	shRNA 2F	CTGCAGGAGAAGCTTTTTG
	EFEMP2	AATTCAAAAAGCTTCTCCTGCAGTGATATTGCTCGAGC
	shRNA 2R	AATATCACTGCAGGAGAAGC
	Scrambled	ACCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAG
	shRNA	GGCGACTTAACCTTAGGTTTTTGAATTC

Abcam), anti-AKT1 (phospho T308) (ab38449; Abcam) and anti-GAPDH (#ab181602; Abcam). This was followed by incubation with the appropriate secondary antibodies for 1 h at room temperature. Protein bands were visualized using an ECL assay kit (Beyond time) and an LAS3000® Luminescent image analyzer (Fujifilm, Tokyo, Japan).

Tumorigenesis test *in vivo*

Four weeks old Female Bal b/c nude mice were obtained from SLAC Laboratory Animal Company (Shanghai, China). All the protocol was approved by the Institutional Animal Care and Use Committee of Shanghai University of Medicine and Health Sciences. Hela cells (5×10^6 cells/mouse) from different groups were injected subcutaneously into the right forelimb axillary of nude mice to generate tumors. The mice were sacrificed after 28 days.

Statistical analyses

All the results represented three or more independent experiments, with the data expressed as the mean ± SD. Differences between the control and treatment groups were analyzed using Student's t-test in SPSS 17.0 and were described in the figure legend for each figure. Differences were considered statistically significant at $P < 0.05$.

Results

Genes associated with extracellular matrix were significantly down-regulated in LTSs patients

We analyzed the 5-year survival rate of cervical cancer in United States from 1975 to 2007 based on the database of Cancer Statistics [27,28]. The 5-year survival rate of total tumors was significantly increased during the 30 years (Figure 1A). However, the 5-year survival rate of cervical cancer was not dramatically augmented. On the contrary, the 5-year survival rate is obviously reduced in black women (Figure 1B). Fortunately, the patients survive time was over five years in more than 50% of cervical cancer patients. We collected the data of cervical cancer patients from TCGA database, including LTSs group and STSs group (Figure 1C), and then compared genes expression. Compared with the STSs patients group, the expression of several genes regulated by TP53 was significantly enhanced the LTS patients group (Figure 1D). However, the several ECM related genes were significantly decreased in the LTS patients compared with the STSs patients group (Figure 1E). Given that great number of studies has focus on the area TP53 and tumor, therefore, we further investigated the gene related to ECM in cervical cancer.

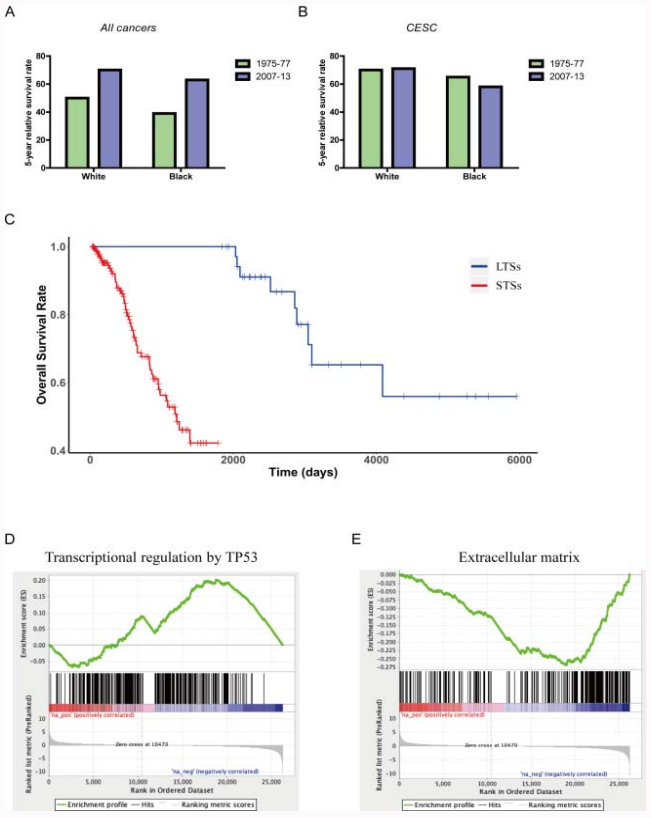

Figure 1: Genes associated with cervical cancer LTSs. A) and B) 5-year Survival rate analysis in United States from 1975 to 2007 C) The cervical patients in TCGA dataset were divided into LTSs group and STSs group. D) ESEA associated with Transcriptional regulation by TP53 between LTSs group and STSs group. E) ESEA associated with Extracellular matrix between LTSs group and STSs group.

EFEMP2 knockdown inhibits the cell proliferation of cervical cancer

Screening the ECM gene, we found a very interesting gene *EFEMP2*, an oncogene and potential biomarker in colorectal cancer. To investigate the association between *EFEMP1* expression, *EFEMP2* expression and the survival rate of cervical cancer, we analyzed the data in TCGA project (Cervical Squamous Cell Carcinoma (CESC)). We discovered that *EFEMP1* expression has no obvious influence;

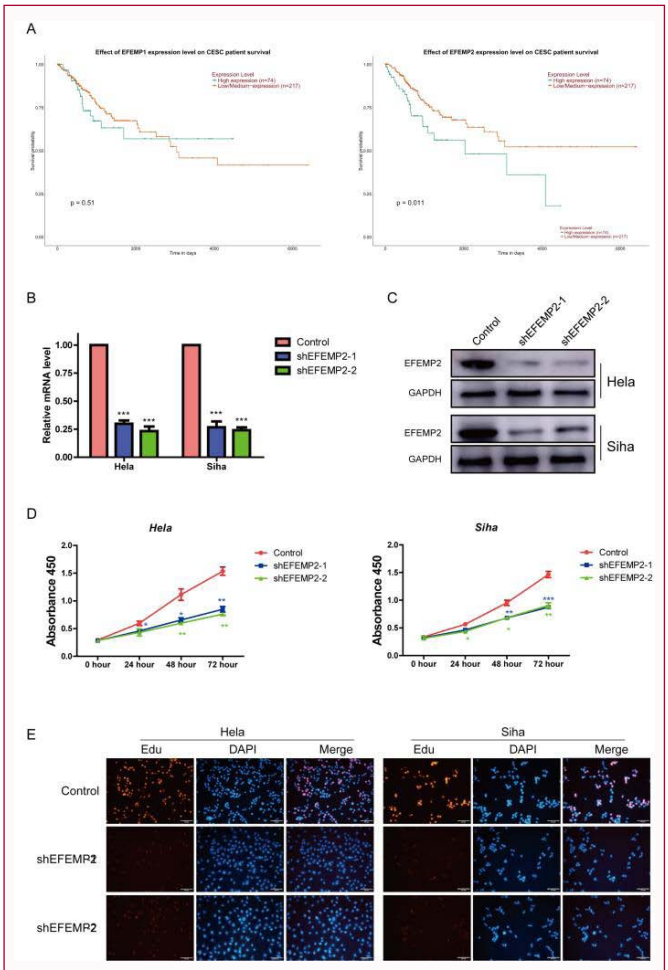

Figure 2: *EFEMP1* KD represses cervical cancer cell proliferation. A) Survival analysis according to gene expression in patients with CESC, who were stratified into high and low expression groups according to median values for *EFEMP1* and *EFEMP2*, with the Kaplan-Meier curve and log-rank test. B and C) qRT-PCR and western blot analysis of *EFEMP2* expression in Control and *EFEMP2* KD cervical cancer cells. D) CCK-8 assay of Control and *EFEMP2* KD cervical cancer cells. E) Ki67 expression in Control and *EFEMP2* KD cervical cancer cells, as determined by IF staining. Bars =200 μm. qRT-PCR data are presented relative to controls as the mean ± SEM (n=3). GAPDH was used as the loading control in western blotting.

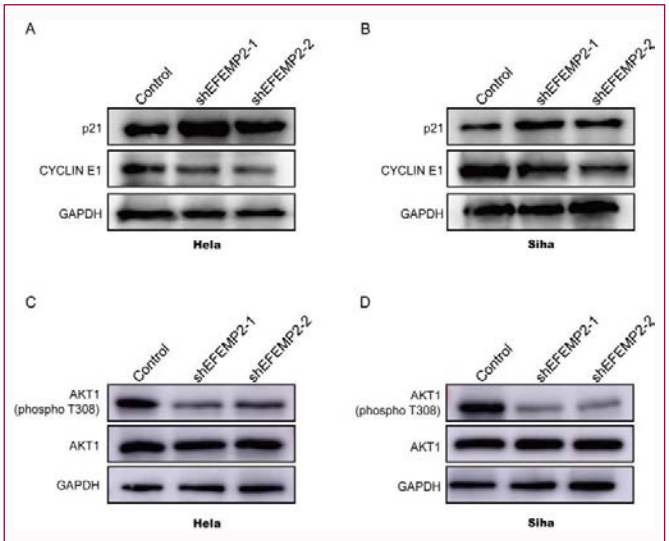

Figure 3: *EFEMP1* KD inhibits cervical cancer cell proliferation through AKT signaling. A and B) Protein level of CYCLIN E1 and p21 in Control and *EFEMP2* KD cervical cancer cells. C and D) Protein level of AKT and AKT (phosphor T308) in Control and *EFEMP2* KD cervical cancer cells.

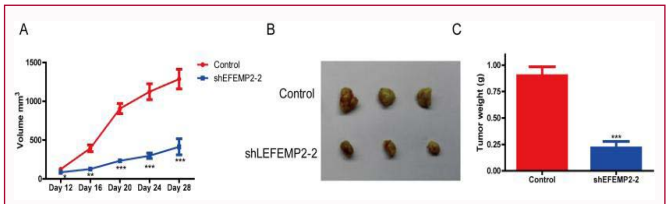

Figure 4: *EFEMP1* KD inhibits HCC cell proliferation *in vivo*. A) The dynamics of tumor development in mice after treatment with Control and *EFEMP2* KD cervical cancer cells. The tumor volumes were measured as follows: Volume=length*width*width/2. Measurements were taken at the indicated time points. I (B) mages of tumors derived from the Control and *EFEMP2* KD cervical cancer cells C) Tumor weights were measured in mice after treatment with Control and *EFEMP2* KD cervical cancer cells.

however, the survival time of the patients with high-level of *EFEMP2* remarkably decreased (Figure 2A). Then, we construct two shRNA-sh*EFEMP2*-1 and sh*EFEMP2*-2 to knockdown *EFEMP2* expression in cervical cancer cell lines. These two shRNA have remarkable knockdown effect in these two cell lines on both mRNA and protein level (Figure 2B, 2C). Previous research demonstrates that *EFEMP2* promotes the proliferation of glioma cells [24]. Therefore, we focus *EFEMP2* function on the proliferation of cervical cancer. The proliferation ability of the cervical cancer cells was significantly reduced when *EFEMP2* knocked down (Figure 2D). The level of Edu, a proliferation marker, also markedly decreased (Figure 2E). Thus, these results imply that *EFEMP2* influences on the proliferation of cervical cancer cells.

EFEMP2 knockdown inhibits the proliferation via suppressing AKT signaling

We then examined several genes related to cellular proliferation. The expression of CYCLIN E1 was markedly reduced after *EFEMP2* knockdown, while the expression of p21/WAF1/Cip1, a cell cycle G1 regulating protein, was markedly increased (Figure 3A, 3B). It is reported that CYCLIN E1 and p21/WAF1/Cip1 were regulated by AKT signaling. Interestingly, we found that the level of AKT activation form (T308 phosphorylated) was decreased markedly (Figure 3C, 3D). Together, these data suggest that *EFEMP2* regulates the proliferation of cervical cancer cells through inhibiting AKT pathway.

EFEMP2 knockdown inhibits tumor growth of cervical cancer *in vivo*

In order to further confirm *EFEMP2* oncogenic functions in cervical cancer, we performed the tumorigenesis test *in vivo*. Compared with the control group, the size of xenograft was significantly decreased in *EFEMP2* knockdown group (Figure 4A). Similarly, compared with the control group, the weight of xenograft was significantly decreased in *EFEMP2* knockdown group (Figure 4B, 4C). Collectively, these results suggest that *EFEMP2* knock down can inhibit the proliferation of cervical cancer cells *in vivo*.

Discussion

Cervical cancer is the most common gynecological tumor worldwide and is one of the leading causes of cancer related death among females [2]. After decades of constant efforts, the 5-year survival rate of breast cancer, the most common cancer in females, has been greatly improved, but the 5-year survival rate of cervical cancer has not progressed significantly [27,28] (Figure1A, 1B). One possible

cause of this strange phenomenon is that large-scale screening of cervical cancer could detect many people with early cervical lesions in developed countries such as the Unite States. After simple surgery therapy, these people will be cured and could not further develop cervical cancer, and will not be included in patients with cervical cancer. Therefore, the patients who are counted in cervical cancer may be more serious and the 5-year survival rate is difficult to be improved [29,30]. Additionally, the choices for cervical cancer treatment are very limit, including surgery, chemotherapy and radiotherapy, lacking effective targeted drug treatment [31]. Our study found that compared to patients with STS cervical cancer, genes regulated by p53 were significantly increased. It is reported that Nutlin-3 can activate p53 and inhibit cell proliferation and tumor growth by activating p21 [32,33]. Therefore, Nutlin-3 may be a promising drug for cervical cancer treatment.

In this study, we also demonstrated that ECM related genes were significantly down-regulated in STSs, suggesting that they play an important role in the progression of cervical cancer. Previous studies have elucidated that ECM genes play extremely important roles in the formation and progression of tumors. For example, knock down COL1A2 suppressed gastric cancer cell proliferation and invasion through regulating the PI3K-AKT signaling [34]. THBS2 regulates endothelial cell migration and apoptosis *via* inhibiting VEGF and nitric oxide [35-37]. These studies, together with our work, reveal the critical role of ECM in tumor formation. In addition, ECM genes are important molecular targets for tumor diagnosis and treatment. For example, through targeting matrix metalloproteinase-9, asprin could inhibit prostate cancer invasion [38,39]. Therefore, these ECM genes may also be potential molecular therapeutic targets for cervical cancer.

We also demonstrated that *EFEMP2* knockdown could inhibit the growth ability of cervical cancer cells. Previous studies have found that *EFEMP2* plays important roles in breast cancer and gliomas [24]. Taken together, *EFEMP2* might be a pan-cancer oncogene. Meanwhile, we also proved that the high expression of *EFEMP2* is significantly negatively correlated with the 5-year survival of cervical cancer patients. As a secreted protein, *EFEMP2* can be detected conveniently in serum. Therefore, it may be a potential diagnostic indicator for the prognosis of cervical cancer. Previous studies have found that *EFEMP2* could be a molecular marker for the diagnosis of colorectal cancer [23]. Thus, *EFEMP2* may be a promising molecular marker for pan-tumor diagnosis. In addition, we revealed that inhibition of *EFEMP2* suppressed cervical cell proliferation via regulating AKT pathway. In fact, inhibition of the AKT pathway could be a potential method for cervical cancer treatment and the AKT pathway inhibitors could be potential drugs for cervical cancer [40,41].

In conclusion, the study here compared genes expression in LTSs and STSs of cervical cancer, and found that p53 regulating-genes were significantly up-regulated and ECM related genes were remarkably down-regulated in LTSs. Furthermore, *EFEMP2* not only affects the proliferation of cervical cancer cells by regulating the AKT pathway, but also could be a promising molecular marker for prognosis prediction of cervical cancer. Our study reveals the factors affecting the survival of cervical cancer patients, and deepens the understanding of the molecular mechanism of cervical cancer, and has provided a promising method for the clinical diagnosis and drug treatment of cervical cancer.

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Authors Contribution

J.W.S. and Z.P.N. designed this research. Y.F.L., H.Y.P. and Z.J. performed the experiments. Y.F.L. and J.W.S. analyzed the data and compiled the figures. Y.F.L. and J.W.S. wrote and revised the manuscript. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research.

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