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Extracellular matrix metalloproteinase inducer (EMMPRIN) remodels the extracellular matrix through enhancing matrix metalloproteinases (MMPs) and inhibiting tissue inhibitors of MMPs expression in HPV-positive cervical cancer cells

Q. Xu[1*], X. Cao[2*], J. Pan[3], Y. Ye[4], Y. Xie[4], N. Ohara[5], H. Ji[1]

[1] Department of Gynecology Oncology, Fujian Provincial Cancer Hospital, Teaching Hospital of Fujian Medical University, Fuzhou
[2] Department of Radiation Oncology, Cancer Center, Sun Yat-sen University, Guangzhou
[3] Department of Radiation Oncology, Fujian Provincial Cancer Hospital, Teaching Hospital of Fujian Medical University, Fuzhou
[4] Department of Research Center, Fujian Provincial Cancer Hospital, Teaching Hospital of Fujian Medical University, Fuzhou (China)
[5] Department of Obstetrics and Gynecology, Sanda Municipal Hospital, Sanda (Japan)

Summary

Purpose of investigation: To study the expression of extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinases (MMPs), and tissue inhibitors of MMP (TIMPs) in uterine cervical cancer cell lines in vitro. *Materials and Methods:* EMMPRIN, MMPs, and TIMPs expression were assessed by Western blot and real-time RT-PCR from cervical carcinoma SiHa, HeLa, and C33-A cells. *Results:* EMMPRIN recombinant significantly increased MMP-2, MMP-9 protein and mRNA expression in SiHa and Hela cells, but not in C33-A cells by Western blot analysis and real-time RT-PCR. EMMPRIN recombinant significantly inhibited TIMP-1 protein and mRNA levels in SiHa and Hela cells, but not in C33-A cells. There was no difference on the TIMP-2 expression in those cells with the treatment of EMMPRIN recombinant. EMMPRIN RNAi decreased MMP-2 and MMP-9 and increased TIMP-1 expression in SiHa and HeLa cells, but not in C33-A cells. There was no change on the expression of TIMP-2 mRNA levels in SiHa, HeLa and C33-A cells transfected with siEMMPRIN. *Conclusions:* EMMPRIN may induce MMP-2 and MMP-9, and downregulate TIMP-1 in HPV-positive cervical cancer cells in vitro.

Key words: Extracellular matrix metalloproteinase inducer (EMMPRIN); Matrix metalloproteinases (MMPs); Tissue inhibitors of MMP (TIMP); Cervical carcinoma.

Introduction

Uterine cervical carcinoma is the second most common malignant tumor in women worldwide, corresponding annually to 16% of all cases of tumors in women [1]. It is the most common gynecological malignancy in China and its incidence has recently increased.

Many risk factors have been demonstrated to influence cervical carcinoma, such as HPV infection, an early onset of sexual activity, parity, pregnancy, immunosuppression, and recent sexual partners [2]. Although cervical cancer can be treated with radical surgery with or without radiotherapy and /or chemotherapy, some patients with high risk factors will still have an unfavorable prognosis. The leading cause of cervical cancer death is not the tumor itself, but its metastasis to lymph nodes and distant organs. Therefore, new strategies, such as immunotherapy and molecular-targeted therapy, may prove useful in improving the prognosis of cervical cancer patients. Cervical cancer provides a useful model to study the relationship of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs) to tumor behavior [3].

Tumor invasion and metastasis are key steps in the progression of cancer and involve the degradation of basement membranes (BM) and subsequent remodeling of the extracellular matrix (ECM) [4]. MMPs are thought to play a central role in ECM turnover and degradation. MMP-2 and MMP-9 are found abundantly in cancer tissues. There is growing evidence of their role in tumor progression. MMP-2 (72-kDa type IV collagenase/gelatinase A) and MMP-9 (92-kDa type IV collagenase/gelatinase B) were of particular importance in tumor progression because they are capable of cleaving type IV collagen, the major collagen of the BM [5]. During the last decades, the progress in research showed overexpression of MMP-2 and MMP-9 in cervical cancer, suggesting their prognostic value [6].

[*] Contributed equally to this work.

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EMMPRIN/CD147 is a membrane-associated glycoprotein with two extracellular loop structures, which belongs to the immunoglobulin superfamily [7]. EMMPRIN is highly expressed on the cell surface of various tumors [8]. Recent studies reported that EMMPRIN might promote tumor invasion and metastasis via stimulating MMP synthesis in neighboring fibroblasts and enhance angiogenesis via vascular endothelial growth factor [9]. A recombinant EMMPRIN was shown to stimulate cultured fibroblasts to produce MMP-1, MMP-2, MMP-3, and augment the production of MMP-9 in monocytes [10]. Gene silencing of EMMPRIN by small-interfering RNA was demonstrated to hinder lipopolysaccharide-induced monocyte secretion of MMP-9, indicating a predominant role of EMMPRIN in MMP-9 induction [11].

TIMPs are endogenous inhibitors of MMPs and efficiently inhibit the enzymatic activity by binding to the catalytic domain of the MMPs [12]. The C-terminal domain of TIMPs is important for the binding to MMPs, thereby regulating the MMP activation process. Substantial evidence suggests the importance of the MMPs/TIMPs ratio in tumor tissues [13]. Many studies have shown a 1:1 ratio of MMPs: TIMPs in early cervical cancers. It suggested that tumor progression may select for cells expressing MMPs and do not express TIMP messages by promoting tumor cell growth.

The dysregulation of the ECM remodeling may play a role in the invasion and metastases of cervical carcinoma. However, it remains unknown whether EMMPRIN acts to regulate ECM remodeling through modulating the expression of MMPs and TIMPs in cervical carcinoma cells and whether it is related to HPV infection.

The present study was designed to elucidate the expression of the ECM components, including EMMPRIN, MMPs, and TIMPs in vitro. The authors have detected the expression of EMMPRIN, MMP-2, MMP-9, TIMP-1, and TIMP-2 in cultured cervical carcinoma cell lines (HeLa, SiHa, and C33-A cells). To explore the role of EMMPRIN in tumor invasion and metastases in three lines of cervical carcinoma cells, the authors examined the effects of EMMPRIN recombinant and RNA interference (RNAi) of EMMPRIN on the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 at mRNA and/or protein levels in those cells.

Materials and Methods

Cell culture

The present study was approved by the Ethics Committee of Fujian Provincial Cancer Hospital, Affiliated Fujian medical university in China. SiHa, HeLa, and C33-A cells were purchased from a Chinese cell bank. Cells were plated in six-well plates at approximately 2×10^6 viable cells per well in one ml of Dulbecco modified Eagle medium containing 10% fetal bovine serum and cultured at 37°C in an atmosphere of 5% CO_2-95% air. Cells were allowed to attach for 24 hours and then incubated in a serum-free medium for 16 to 18 hours. Uterine leiomyoma tissues were digested in 0.2% collagenase (wt/vol) at 37°C for three to five hours. The leiomyoma cells were collected by centrifugation at 460 × *g* for five minutes and washed three times with PBS containing 1% antibiotic solution. The isolated leiomyoma cells were plated at densities of approximately 1×10^6 cells/dish in ten-cm^2 culture dishes. The isolated leiomyoma in culture dishes were subcultured at 37°C for 120 hours in a humidified atmosphere of 5% CO_2-95% air in phenol red-free DMEM supplemented with 10% fetal bovine serum.

Western blot analysis

Samples were lysed with a radioimmunoprecipitation assay (RIPA) buffer containing 100 M phenylmethylsulfonyl fluoride, and the protein concentrations were determined using a BCA kit. Equal amounts of total protein were separated on 12% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were immunoblotted with the appropriate primary antibody diluted in tris-buffered saline (TBS) buffer containing 0.05% Tween-20 and 5% non-fat dry milk at room temperature for two hours. The primary antibodies were used: EMMPRIN (sc-9753), MMP-2 (sc-373914), MMP-9 (sc-6841), TIMP-1 (sc-6832), TIMP-2 (sc-9905), and pan-actin (MS-1295-P0). After extensive washing, the membranes were incubated with an HRP-labeled antibody for one hour at room temperature. Protein bands were revealed by ECL detection. EMMPRIN recombinant protein (972-EMN-050) (50 ug) was provided.

Real-time reverse transcription polymerase chain reaction analysis

Total RNA was isolated using the RNeasy Protect kit. First strand cDNA was synthesized from two-μg total RNA using an Omniscript RT Kit. The primer of MMP-2, MMP-9, TIMP-1, TIMP-2, and GADPH equipped with Light Cycler-Primer Set were used. Real-time RT-PCR was performed in a LightCycler platform. Each sample was analyzed in duplicate in three independent real-time RT-PCR assays. Cycling conditions included the incubation at 95°C for ten minutes, and 35 cycles of 95°C for ten seconds, 68°C for ten seconds and 72°C for 16 seconds. Synthesis of a DNA product of the expected size was confirmed by melting curve analysis using the LightCycler software.

Small interfering RNA (siRNA) targeting of EMMPRIN genes

The siRNA for human EMMPRIN mRNA target sequences was synthesized by NIPPON EGT. The EMMPRIN-specific siRNA sequence was AGUCGUCAGAACACAUCAAdTdT. Non-specific siRNA [(UUCUCCGAACGUGUCACGU)d(TT)] which does not match to any sequence of the human genome was used as a negative control. After reaching approximately 70% confluence, cultured cervical carcinoma cells (SiHa, HeLa, and C33-A) were transfected with EMMPRIN siRNA for 48 hours using siScreen Custom Plate. At the end of transfection, the medium was replaced with serum-free, phenol red-free DMEM for 24 hours for Western blot analysis and/or real-time RT-PCR analysis.

Statistical analysis

The data were expressed as the mean ± SD from at least three independent experiments. Statistical significance was determined using Student's *t*-test and one-way ANOVA. A difference with a $p < 0.05$ was considered statistically significant.

Results

EMMPRIN expression in three cervical cancer cell lines by western blot analysis

As shown in Figure 1a,b, EMMPRIN protein levels in HeLa, SiHa, and C33-A cell lines were positively expressed compared with control by Western blot analysis ($p < 0.05$) (Fig. 1a, b). Primary cultured leimyoma cells were used as positive control. Western blot analysis demonstrated that the transfection of cultured cervical cancer cells with siRNA

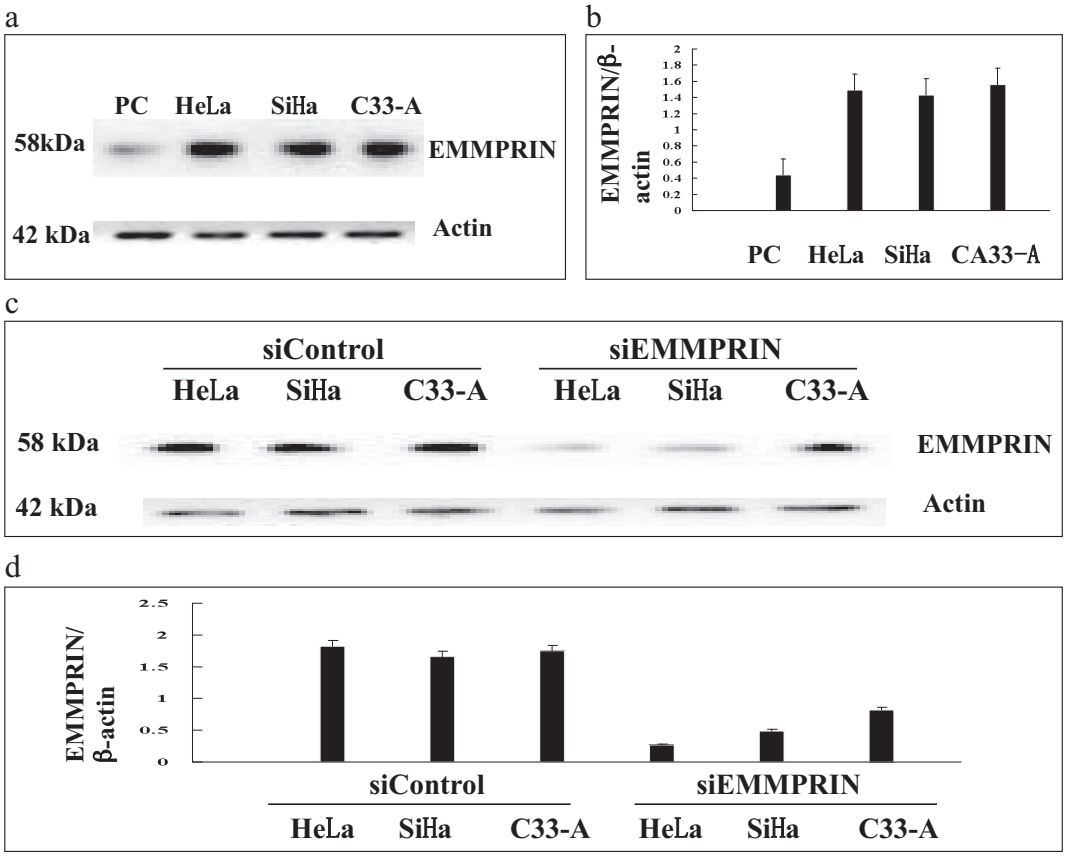

Figure 1. — The expression of EMMPRIN in HeLa, SiHa, and C33-A cervical carcinoma cells. EMMPRIN (a, b): pro- tein levels in HeLa, SiHa, and C33-A cervical carcinoma cells as assessed by Western blot analysis. Cultured cervical carcinoma cells were transfected with EMMPRIN si-RNA for 48 hours. EMMPRIN protein content was suppres-sed approximately 60-80%. (c, d) $p < 0.05$ compared with the cells transfected with non-specific siRNA control (siControl) cells by Western bolt. *$p < 0.05$ *vs.* positive control (leiomyoma cells) or siControl cells.

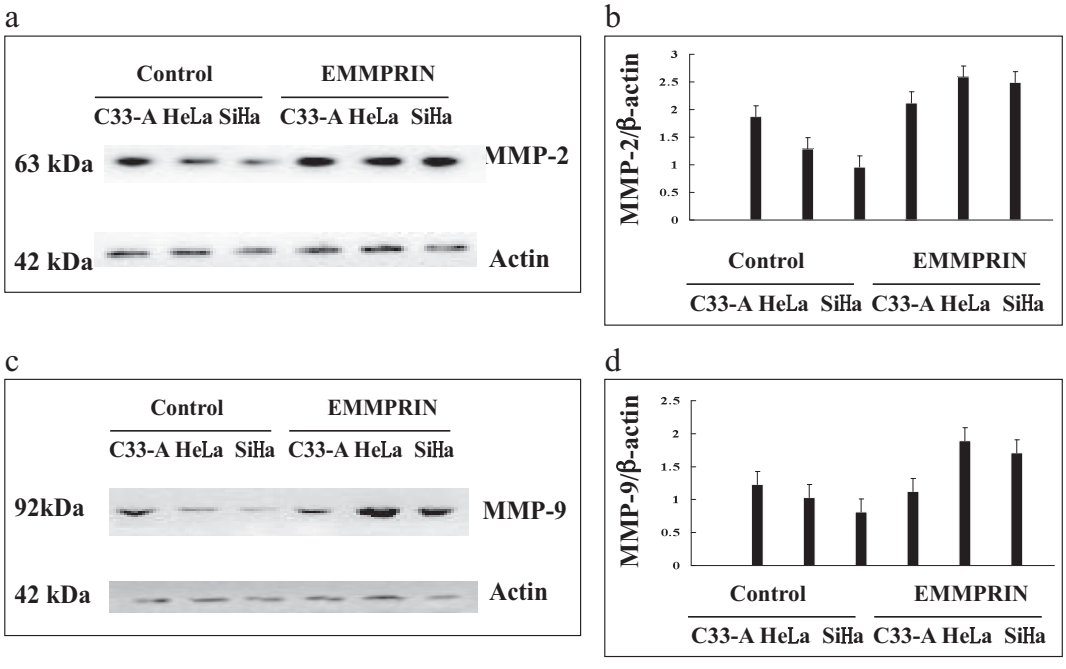

Figure 2. — The expression of MMP-2 and MMP-9 with the treatment of EMMPRIN recombinant MMP-2 (a,b) and MMP-9 (c,d) protein levels were enhanced in HeLa and SiHa cells but not in C33-A cervical carcinoma cells when treated with EMMPRIN recombinant as assessed by Western blot analysis. *$p < 0.05$ *vs* control (untreated cervical carcinoma cells).

EMMPRIN (siEMMPRIN) suppressed EMMPRIN protein content compared with the cells transfected with non-specific siRNA control (siControl) cells with the inhibition rate being approximately 60-80% (Figure 1c, d) ($p < 0.05$).

Effects of EMMPRIN recombinant on the ECM components in cultured cervical carcinoma cell lines

The present results showed that by treatment with EMMPRIN recombinant MMP-2 (Figure 2a, b) and MMP-9

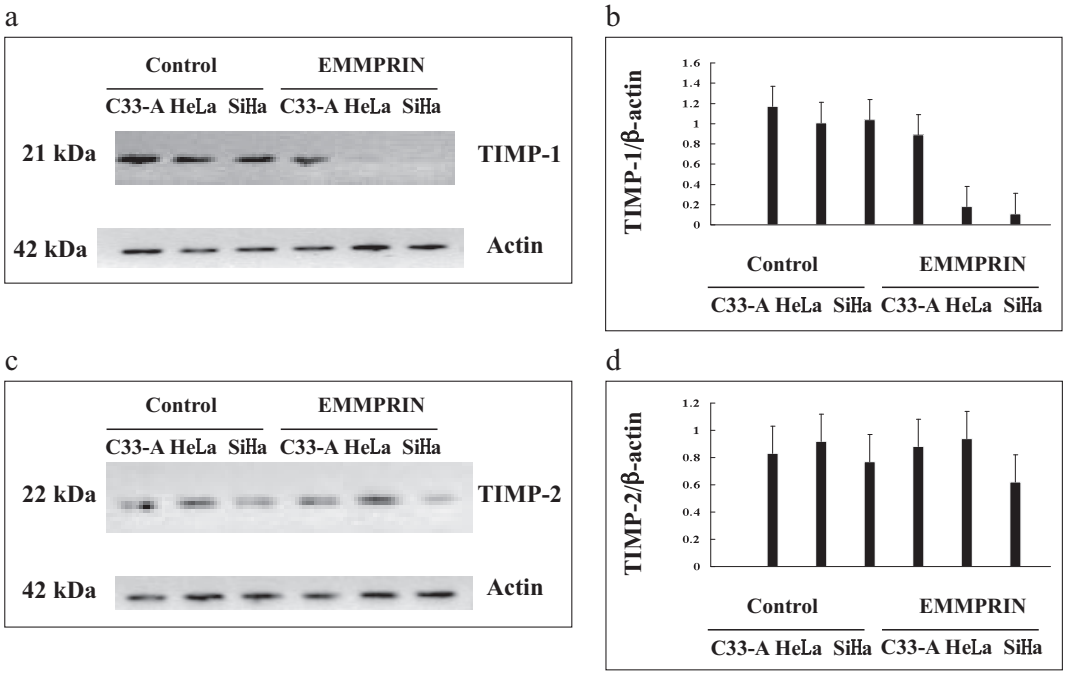

Figure 3. — The expression of TIMP-1 and TIMP-2 with the treatment of EMMPRIN recombinant. TIMP-1 (a,b) protein levels were decreased in HeLa and SiHa cells but not in C33-A cervical carcinoma cells when treated with EMMPRIN recombinant compared with control, as assessed by Western blot analysis. There was no change in the expression of TIMP-2 protein levels compared with control by Western blot analysis. *$p < 0.05$ *vs* control (no-treated cervical carcinoma cells).

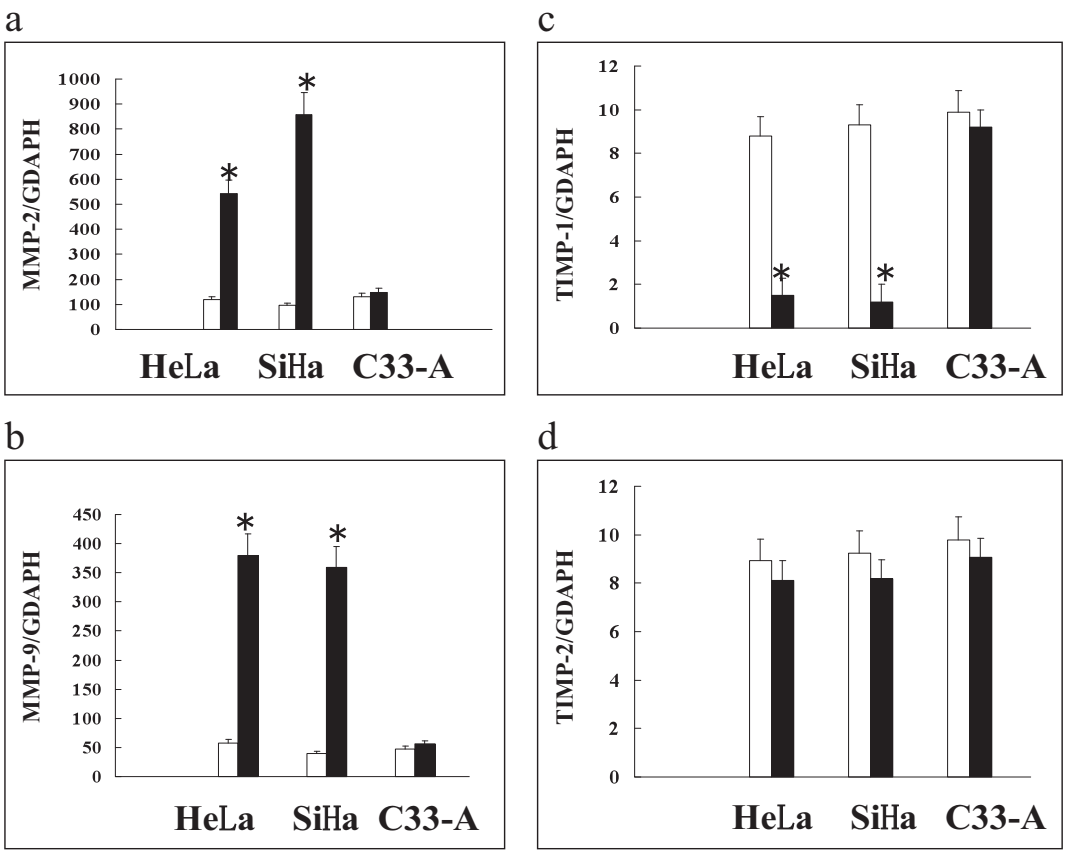

Figure 4. — Effects of EMMPRIN recombinant on MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in cultured HeLa, SiHa, and C33-A cervical carcinoma cells. Cultured cervical carcinoma cells were treated with EMMPRIN recombinant. Enhanced MMP-2 (a) and MMP-9 (b) mRNA levels and decreased TIMP-1 mRNA levels in cultured HeLa and SiHa cells but not in C33-A cells treated with EMMPRIN recombinant. There was no difference on the expression of TIMP-2 in cultured three cervical carcinoma cell lines treated with EMMPRIN recombinant. *$p < 0.05$ *vs* control (untreated cervical carcinoma cells).

(Figure 2c, d) protein levels were significantly ($p < 0.05$) enhanced in HeLa and SiHa cells but not in C33-A cells compared with controls. On the other hand, in EMMPRIN recombinant group TIMP-1 (Figure 3a, b) protein levels were significantly ($p < 0.05$) decreased in HeLa and SiHa cells but not in C33-A cells. However, there was no signif-

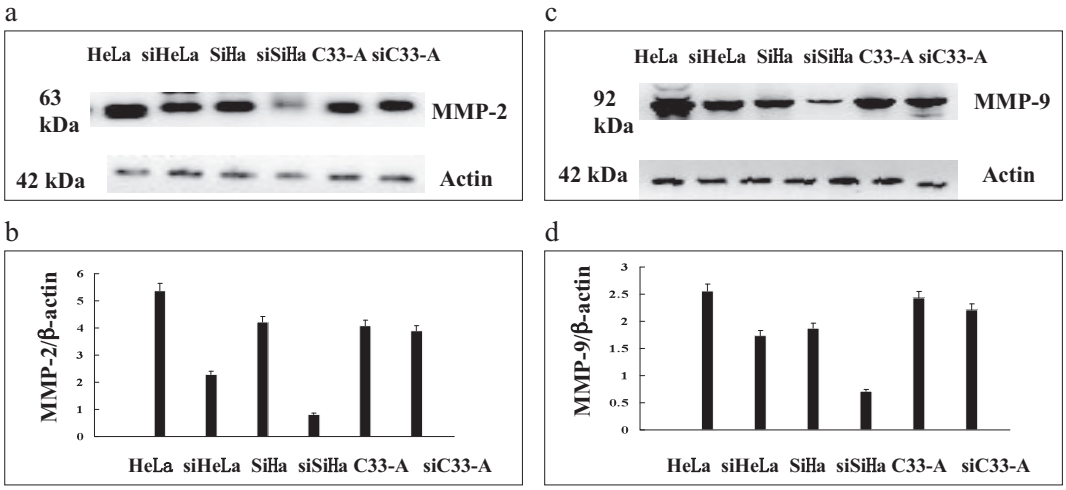

Figure 5. — Effects of RNA interference of EMMPRIN on the expression of MMPs protein levels in cultured cervical carcinoma cells. Reduced MMP-2 protein content (a, b), and reduced MMP-9 protein content (c, d) in cultured HeLa and SiHa cells by RNA interference of EMMPRIN. There was no difference on the expression of MMP-2 and MMP-9 in cultured C33-A cells by RNA interference of EMMPRIN. *$p < 0.05$ *vs* siControl cells.

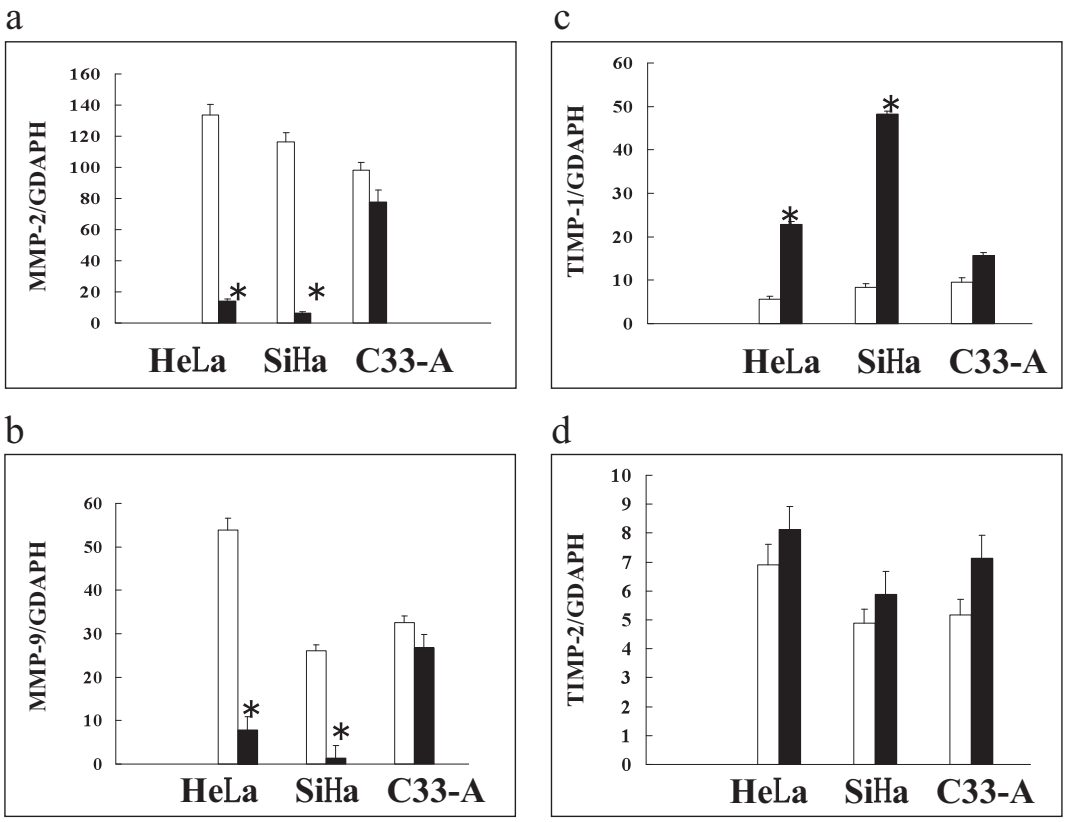

Figure 6. — Effects of RNA interference of EMMPRIN on MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression in cultured HeLa, SiHa, and C33-A cervical carcinoma cells. Cultured cervical carcinoma cells were transfected with siControl and/or EMMPRIN siRNA for 48 hrs. Reduced MMP-2 (a) and MMP-9 (b) m-RNA levels and increased TIMP-1 m- RNA levels in cultured HeLa and SiHa cells but not in C33-A cells by RNA interference of EMMPRIN. There was no difference on the expression of TIMP-2 in cultured 3 cervical carcinoma cell lines by RNA interference of EMMPRIN. *$p < 0.05$ *vs* siControl cells.

icant difference of TIMP-2 protein levels (Figure 3c, d) among three cervical cancer cell lines.

In addition, mRNA levels of MMP-2 and MMP-9 were significantly ($p < 0.05$) augmented in HeLa and SiHa cells but not in C33-A cells with the treatment of EMMPRIN recombinant (Figure 4a, b) by using real-time RT-PCR. In contrast, TIMP-1 mRNA levels was significantly ($p < 0.05$) decreased in HeLa and SiHa cells but not in C33-A cells with the treatment of EMMPRIN recombinant (Figure 4c). There was no change on the TIMP-2 mRNA levels in three cervical cancer cell lines (Figure 4d).

Effects of EMMPRIN RNA interference on the ECM components in cultured cervical carcinoma cell lines

The authors transfected siRNA targeted to EMMPRIN and tested its effect on EMMPRIN, MMP-2, MMP-9,

TIMP-1, and TIMP-2 protein and/or mRNA levels. Si-EMMPRIN was shown to significantly ($p < 0.05$) decrease MMP-2 (Figure 5a, b) and MMP-9 (Figure 5c, d) protein contents in HeLa and SiHa cells but not in C33-A cells.

On the other hand, mRNA levels of MMP-2 and MMP-9 were significantly ($p < 0.05$) decreased in HeLa and SiHa cells but not in C33-A cells transfected with siEMMPRIN (Figure 6a, b) by using real-time RT-PCR. In contrast, TIMP-1 mRNA levels was significantly ($p < 0.05$) increased in siEMMPRIN cells compared with siControl cells in HeLa and SiHa cells but not in C33-A cells (Figure 6c). RNAi of EMMPRIN resulted in no difference of TIMP-2 mRNA levels in three cervical cancer cell lines (Figure 6d).

Discussion

In the present study, the authors have demonstrated that EMMPRIN, MMPs (MMP-2, MMP-9), and TIMPs (TIMP-1, TIMP-2) were expressed in HeLa, SiHa, and C33-A cervical carcinoma cell lines. Furthermore, a series of studies of EMMPRIN recombinant and RNAi experiments indicated an essential role of EMMPRIN in the regulation of the expression of MMPs and TIMPs in three cultured cervical carcinoma cells.

EMMPRIN is a highly glycosylated cell surface transmembrane protein and expressed as a glycoprotein with a molecular mass of 44-66 kDa due to different degrees of glycosylation of the native protein (-30 kDa) [14]. Li *et al.* evaluated that EMMPRIN overexpression was found in 28 types of cancers from 14 organs and the haematological system [15]. EMMPRIN appears to participate in the induction of MMP, such as MMP-1, 2, 3, 9, 14, and 15 in fibroblasts surrounding tumor cells [16].

In this research, the authors demonstrated that EMMPRIN was highly expressed in HeLa, SiHa, and C33-A cells. EMMPRIN recombinant can significantly enhance the expression of MMP-2 and MMP-9 in HeLa and SiHa cells, but not in C33-A cells. In the previous research, the authors have reported that EMMPRIN may upregulate MMPs (MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9) and downregulate TIMPs (TIMP-1 and TIMP-2) and collagens (collagen type I and III) in cultured leiomyoma cells [17]. In addition, it was shown that downregulation of EMMPRIN inhibited secretion of MMP-9 and MMP-2 in the cultured T24 cells [18]. Kapral *et al.* reported that phytic acid can decrease in MMP-2 transcript level, but not MMP-9 and resulted in a strong increase in both TIMP-1 and TIMP-2 expression in colon cancer cells [19].

Zou *et al.* demonstrated that inhibition of EMMPRIN gene expression via RMAi could reduce tumor cell invasion and tumorigenicity in HO-8910pm EOC cells [20]. Transfection of anti-sense RNA of EMMPRIN into hepatocelluar carcinoma cells has been reported to decrease the secretion of MMP-9 and inhibit tumor cells for invasion and metastasis [21]. The present authors assessed the role of the endogenous MMP inhibitor on the anti-invasive effects in HeLa, SiHa, and C33-A cells. They transfected siEMMPRIN in HeLa, SiHa, and C33-A cells and showed that RNAi of EMMPRIN resulted in a significant inhibition of MMP-2 and MMP-9 protein contents and mRNA levels in HeLa and SiHa cell lines but not in C33-A cells.

In contrast, the present authors demonstrated that EMMPRIN recombinant inhibited TIMP-1 protein and mRNA levels in HeLa and SiHa cell lines but not in C33-A cells. There was no difference in TIMP-2 expression in those cells. TIMP-1 but not TIMP-2 mRNA levels were significantly increased in siEMMPRIN cells compared with siControl cells in HeLa and SiHa cells but not in C33-A cells. Similar to MMP-1 and MMP-9, TIMP-1 and TIMP-2 have activator protein-1 (AP-1) binding sites in their promoters, and the transcription of TIMP-1 is AP-1 dependent [22]. Therefore, it is suggested that the different responses of TIMP-1 and TIMP-2 expression to EMMPRIN in cervical cancer cell lines may be attributable to the different contributions of AP-1 to the transcriptional activation. Reddy *et al.*, reported that the IL-18 induction of MMP-9 was mediated in part via EMMPRIN and through JNK- and ERK-dependent AP-1 activation and p38 MAPK-dependent NF-κB activation [23]. One of the explanation of the different responses of the TIMP-1 and TIMP-2 expression when treatment with EMMPRIN could be that EMMPRIN may induced MMP-9 through JNK- and ERK-dependent AP-1 activation and inhibited TIMP-1 expression in cultured Hela and Siha cell lines but not in C33-A cells. In a future study, the present authors should detect whether or not AP-1 was induced by EMMPRIN through JNK- and ERK-dependent pathway in HPV-positive cervical cancer cell lines.

The present study suggested a possible association of EMMPRIN with upregulation of MMP-2 and MMP-9 and downregulation of TIMP-1, but not TIMP-2 in HeLa and SiHa cell lines. Strong epidemiologic evidence has linked infection with 'high-risk' HPVs, HPV-16, and -18, which infect the anogenital mucosa that progress to the development of cervical cancer. Therefore, the present authors chose SiHa and HeLa cells for this study, which are infected with these virus types. They also included an HPV-negative cervical cancer cell line (C33-A). It is postulated that EMMPRIN may be involved in the MMP-dependent pathway through upregulating MMP-2 and MMP-9 and downregulating TIMP-1 in HPV-positive cervical cancer. Del Toro-Arreola *et al.*, have demonstrated that cell surface expression of MHC class I chain-related chain A (MICA) was higher than cell surface expression of MICB in the HPV-positive cell lines; in contrast, HPV-negative cells expressed lower levels of MICA. Sustained over-expression of MICA at the cell surface of HPV-positive cells, which could promote downregulation of the NK cell functions [24]. However, numerous studies have demonstrated tumor evasion through metalloprotease-induced proteolytic release of MICA and MICB from the cell surface,

which provokes downregulation of NKG2D in NK and T cells. MMP-9 is critically involved in the osteosarcoma-associated proteolytic release of sMICA, which facilitates tumour immune escape [25]. Further study is necessary to elucidate whether EMMPRIN may induce the sMICA expression through NKG2D receptor by MMP-9-dependent pathway in HPV-positive cervical cancer cell lines.

Conclusion

The authors demonstrated the expression of EMMPRIN, MMP-2, MMP-9, TIMP-1, and TIMP-2 in cultured HeLa, SiHa, and C33-A cells. Furthermore, they provided novel evidence that EMMPRIN may play a vital role in upregulating MMP-2 and MMP-9 and downregulating TIMP-1, but not TIMP-2 in HPV-positive cervical cells. Upregulated EMMPRIN expression may contribute to tumorigenesis, tumor growth, and vascular invasion of HPV-positive cervical cancer. These data suggest that EMMPRIN could become a good marker to predict the prognosis of HPV-positive cervical cancer and that it could be a promising target for improved cancer therapy in the future.

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Address reprint requests to:
J. PAN, MD. PhD.
Department of Radiation Oncology
Fujian Provincial Cancer Hospital,
Teaching Hospital of Fujian Medical University
91 Maluding, Fuma Road
Fuzhou, Fujian 350014 (China)
e-mail: 1379423879@qq.com