Original Article
EMMPRIN inhibition suppresses proliferation, invasion and tumourigenicity of acute monocytic leukaemia SHI-1 cells

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Abstract: Acute monocytic leukaemia (AML) is a type of acute myelogenous leukaemia that is characterized by abnormal accumulation of immature myelomonocytic cell lineages in the bone marrow and peripheral blood. EMMPRIN (Basigin) has been implicated in the pathology and physiology of many human cancers. The present study investigated the effects of EMMPRIN on acute monocytic leukaemia SHI-1 cells. Here, we found that inhibiting EMMPRIN by lentiviral shRNA led to decreased SHI-1 cell growth and invasion and reduced MMP-2 and MMP-9 protein expression levels. Furthermore, an in vivo tumourigenicity assay revealed that EMMPRIN knockdown repressed tumour growth in nude mice. Thus, our data demonstrated that inhibiting EMMPRIN expression played an antitumour role in SHI-1 cells, suggesting that selective targeting of EMMPRIN may be a promising therapeutic strategy for AML.

Keywords: AML, SHI-1, inhibition, EMMPRIN, tumourigenesis

Introduction

Acute monocytic leukaemia (AML) is a serious, life-threatening malignancy [1]. Although chemotherapy can induce complete remission in 80-90% of patients with AML in clinics, the relapse rate remains high, with a 5-year event-free survival rate of approximately 50% [2, 3]. Therefore, better understanding the molecular mechanisms underlying AML progression may help to develop novel and effective therapies for this cancer.

EMMPRIN (Basigin) is a highly glycosylated cell-surface transmembrane protein, which plays important roles in spermatogenesis [4], neural network formation [5], embryo implantation [6] and tumour progression [7, 8]. EMMPRIN has been reported to induce expression of MMP-1, MMP-2, MMP-9 and MMP-14 in endothelial cells and peritumoural fibroblasts and promote tumour migration and invasion [9]. EMMPRIN was recently demonstrated to facilitate tumour angiogenesis by increasing VEGF (vascular endothelial growth factor) expression in neighbouring fibroblasts through PI3K-AKT signalling [10, 11]. EMMPRIN is also associated with multidrug resistance in cancer cells through hyaluronan-mediated activating of the ErbB2 signalling pathway [12, 13].

Fu et al. investigated the connection between EMMPRIN expression and clinicopathological characteristics in patients with acute myeloid leukaemia [14]. EMMPRIN expression was significantly higher in patients with acute myeloid leukaemia than in normal controls, and its expression was positively correlated with bone marrow microvessel density and poor overall survival [14]. However, the effects of knockdown EMMPRIN expression by RNA interference in acute monocytic leukaemia SHI-1 cells have not been investigated.

In the present study, we used a lentiviral vector system to introduce short hairpin RNA (shRNA)-triggered RNA interference (RNAi) to knockdown EMMPRIN expression in SHI-1 cells. We further assessed the effects of EMMPRIN inhibition on the growth, invasion and tumourigenicity of SHI-1 cells in vitro and in vivo. The results showed that EMMPRIN was involved in AML development, and targeting EMMPRIN may be a promising gene therapy for this disease.
Material and methods

Cell cultures

Human acute monocytic leukaemia cells (SHI-1) were purchased from Jiangsu Province Blood Institute (Jiangsu, China). SHI-1 cells were cultivated in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% foetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified incubator containing 5% CO₂.

Cell transfection

Based on EMMPRIN’s mRNA sequence in GenBank (NM_001728.3), shRNA oligonucleotides (shRNA) and non-specific oligonucleotides (NC-shRNA) were designed and synthesized by GenePharma (Shanghai, China). The oligonucleotide sequences were 5'-GCACAGUCUUCACUACCGUG-3' (shRNA) and 5'-GAGUGGUACUAUCGUACAC-3' (NC-shRNA). The shRNA and NC-shRNA were cloned into the lentiviral shRNA expression plasmid of pRNAT-U6.2/Lenti (GenePharma; Shanghai, China) and packaged into 293-T cells per the manufacturer’s protocols. Next, approximately 1 ml of the packaged lentivirus was introduced into the SHI-1 cells. Forty-eight hours posttransduction, the transduced SHI-1 cells were selected with G418 (Sigma, USA) at 400 mg/ml for two weeks. The stably transduced SHI-1 cells were verified by quantitative real-time RT-PCR and Western blot.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated by using TRizol reagent (Invitrogen, CA, USA) per the manufacturer’s protocols. Total RNA was then reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan). qRT-PCR was performed using an ABI PRISM 7500 real-time PCR system (Applied Biosystems, CA, USA). The EMMPRIN primers were 5’-CCATGCTGGTCTGCAAGTCAG-3’ (forward) and 5’-CCGTTCATGAGGGCCTTGTC-3’ (reverse). β-actin was used as an internal reference. The β-actin primers were 5’-CTGGACTGGTGAAGGTA-3’ (forward) and 5’-AAGGACTTCCTGTAACAACG-3’ (reverse). The amplification reaction conditions were 95°C for 20 min, followed by 45 cycles at 95°C for 20 sec and 60°C for 30 sec. The relative expression levels of EMMPRIN were normalized to that of the β-actin amplified from the same sample and calculated by the 2^(-∆∆Ct) method [15].

Western blot

Cells were harvested and lysed with ice-cold RIPA buffer (Invitrogen, CA, USA) for 30 min at 4°C. Cell lysates were collected after centrifugation at 12,000 rpm at 4°C for 30 min. Equal amounts of protein from each sample (approximately 30 μg) were separated on 12% SDS-PAGE gels (Beyotime, Shanghai, China) and blotted onto PVDF membranes (Millipore, MA, USA). After blocking with 5% non-fat milk in TBST buffer (Beyotime, Shanghai, China) at room temperature for 2 h, the membranes were cultured with a 1:1000 dilution of anti-EMMPRIN (#ab108317; Abcam, Cambridge, MA, USA), a 1:2000 dilution of anti-β-actin (#ab-6276; Abcam, Cambridge, MA, USA), a 1:1000 dilution of anti-MMP-2 (#ab7033; Abcam, Cambridge, MA, USA) and a 1:500 dilution of anti-MMP-9 (#ab73734; Abcam, Cambridge, MA, USA) antibodies overnight at 4°C, then incubated with 1:5000 dilutions of the secondary antibodies conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, MA, USA) at room temperature for 2 h. Protein bands were detected by using an ECL detection system (Pierce, IL, USA).

Cell proliferation assay

Cell proliferation activity was detected using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) assay in vitro. Briefly, approximately 4,000 SHI-1 cells per well were plated into 96-well plates. After 0, 24, 48, and 72 h of posttransduction, 20 μl of MTT (5 mg/ml) was added to each well and incubated for an additional 6 h at 37°C in a humidified incubator containing 5% CO₂. The medium was then removed, and the formazan crystal was solubilized with 150 μl of dimethylsulfoxide (DMSO; Sigma, USA) solution. Spectrometric absorbance at 480 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

In vitro co-cultured trans-matrigel invasion assay

SHI-1 cells were cocultured with BMSCs to determine their invasion capacity. The invasion assay was performed using 40 μl Matrigel (Becton-Dickinson, USA), and a reconstituted basement membrane was pre-coated on the upper compartment of the Millicell (Millipore, CA, USA) containing an 8 μm pore. Approximately
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600 µl of IMDM with 10% FCS was added to the lower compartment, and 300 µl of the completed medium containing $2 \times 10^5$ SHI-1 cells or/and $2 \times 10^4$ leukaemic BMSCs were seeded to the upper compartment. BMSC conditional supernatant was used to investigate whether conditional supernatant promoted SHI-1 invasion. shRNA or NC-shRNA was added to the cocultured system at a final concentration of 2 µg/ml. After coculturing for 72 hours at 37°C in humidified 5% CO$_2$, cells that migrated to the lower compartment were counted using a blood cell-counting chamber under light microscopy (Leica microscope, Nussloch, Germany).

In vivo tumourigenicity assay

Four-week-old male BALB/c nude mice were obtained from the Laboratory Animals Center of South Medical University (Guangzhou, China). All mice were fed under specific pathogen-free (SPF) conditions at ~22°C under a 12 h light/dark cycle with free access to food and water. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources for the National Research Council and approved by the Animal Ethics Committee of the Second Affiliated Hospital of Nanchang University (Nanchang, China). All mice were pretreated with cyclophosphamide per to the manufacturer’s protocols. Tumour xenografts were established by subcutaneously injecting $5 \times 10^6$ shRNA and NC-shRNA of SHI-1 cells into the right flank of 4-week-old male nude mice. Tumour size was measured using Vernier callipers every 5 days, and the average tumour volume was measured as volume = $1/2 \times (width^2 \times length)$. All mice were euthanized after 35 days postinoculation. The tumour tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for further analysis of EMMPRIN expression by Western blot.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Each assay was conducted and repeated at least three times. All experimental data are presented as the mean ± SD (standard deviation) and were analysed by Student’s t-tests or one-way ANOVA followed by Dunnett’s test. $P<0.05$ was considered statistically significant.
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Results

Lentiviral shRNA targeting EMMPRIN suppressed EMMPRIN expression in SHI-1 cells

The knockdown efficiency of EMMPRIN lentiviral shRNA in SHI-1 cells was first assessed using qRT-PCR assay. Relative EMMPRIN mRNA levels in each stable transfectant was normalized against the mRNA levels of β-actin. As shown in Figure 1A, SHI-1 cells transfected with shRNA showed significantly reduced EMMPRIN mRNA transcription compared with that of NC-shRNA transfectants (P<0.05).

In addition, Western blot analysis showed decreased EMMPRIN protein expression in SHI-1 cells transfected with shRNA (Figure 1B, P<0.05). These results demonstrated that this shRNA effectively downregulated EMMPRIN expression.

Inhibiting EMMPRIN inhibited cell proliferation in vitro

Next, we tested three time points (24, 48 and 72 h) to determine the growth ability of SHI-1 cells after EMMPRIN knockdown. The results showed that shRNA-transfected SHI-1 cells exhibited decreased cell growth rates compared with the NC-shRNA-treated cells (Figure 2, P<0.05). The proliferation inhibition rates of SHI-1 cells treated with shRNA were 12.7% and 27.3% at 48 and 72 h, respectively.

EMMPRIN silencing reduced the SHI-1 cells’ invasive ability in vitro

To examine whether EMMPRIN knockdown in SHI-1 affected its invasive activity, we performed an in vitro Matrigel transwell assay. The results showed that the invasive number of shRNA-treated SHI-1 cells was significantly lower than that of the NC-shRNA-treated cells (Figure 3A, P<0.05).

Since MMP-2 and MMP-9 play important roles in tumour cell invasion [16], we explored EMMPRIN’s effect on silencing MMP-2 and MMP-9 protein expression by Western blot analysis. The expression levels of both MMP-2 and MMP-9 were markedly decreased in shRNA treated-SHI-1 cells compared with NC-shRNA-treated cells (Figure 3B, P<0.05).
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EMMPRIN knockdown restrained tumour growth in vivo

After cell inoculation into male nude mice for 35 days, the tumour tissues were collected and their volume was calculated. In mice inoculated with shRNA, the tumour volumes were reduced compared with those in the mice transplanted with NC-shRNA (Figure 4A, P<0.05). Tumour tissue weight in the shRNA group was markedly lower than in the NC-shRNA group (Figure 4B, P<0.05). In addition, Western blot analysis showed that EMMPRIN protein levels were also less expressed in tumour tissues derived from shRNA-treated SHI-1 cells than in tumours derived from NC-shRNA-treated cells (Figure 4C, P<0.05).

Discussion

EMMPRIN is a transmembrane glycoprotein that is upregulated in various human cancers and plays multiple roles [17-20], such as promoting proliferation, inducing metastasis, facilitating tumourigenicity, inhibiting apoptosis and decreasing chemotherapeutic sensitivity. Although the mechanisms of these phenomena are unclear, EMMPRIN is clearly a major mediator of tumour cell behaviours.

EMMPRIN is enriched in leukaemia, but the potential roles of EMMPRIN in AML remain poorly understood. In this study, we used RNAi technology to construct an EMMPRIN lentiviral shRNA for transfection into acute monocytic leukaemia SHI-1 cells. We found that the shRNA effectively inhibited EMMPRIN expression at the mRNA and protein levels in SHI-1 cells.

We then examined the effect of EMMPRIN inhibition on SHI-1 cell growth. Chen et al. [21] found that EMMPRIN knockdown suppressed proliferation of A375 human malignant melanoma cells. Yang et al. reported that inhibiting EMMPRIN reduced proliferation of human salivary adenoid cystic carcinoma cells [22]. Li et al. showed that downregulating EMMPRIN expression inhibited HT29 cell proliferation [23]. Consistent with the results of previous studies, the proliferation potential of shRNA-treated cells was reduced compared with that of the NC-shRNA-treated cells.

Tumour cell metastasis is the main cause of treatment failure in patients with cancer. MMPs (matrix metalloproteinases), a family of zinc-dependent endopeptidases, play crucial roles in tumour cell invasion and metastasis [16]. Of the MMPs, MMP-2 and MMP-9 are upregulated in cancers and contribute to the metastatic spread of tumour cells [16]. Increasing evidence suggests that MMP expression is correlated with EMMPRIN expression levels in various tumours [24]. Overexpression of EMMPRIN into MDA-MB436 breast cancer cells resulted in increased MMP-2 and MMP-9 expression and enhanced tumour growth [25]. Here, a Matrigel invasion assay indicated that SHI-1 cell invasion was decreased significantly after being transfected with EMMPRIN lentiviral shRNA. MMP-2 and MMP-9 expression were also markedly reduced in shRNA-treated SHI-1 cells. In addition, in vivo tumourigenicity assays revealed that inhibiting EMMPRIN expression suppressed tumour cell growth. Western blot analysis showed that EMMPRIN protein expression was significantly downregulated in tumour tissues derived from shRNA-treated cells. These data strongly demonstrate that EMMPRIN acts as an oncogene in AML.

In summary, our results demonstrate for the first time that EMMPRIN silencing significantly inhibits SHI-1 cell growth, invasion and tumourigenicity.
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genicity, indicating EMMPRIN may be a potential target for AML treatment.

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Disclosure of conflict of interest

None.

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