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Silencing Pin1 Suppresses the Expression and Bioactivity of MMP-9 through NF-κB in Colorectal Carcinoma SW480 Cells

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OBJECTIVE To examine the effect of Pin1 on the expression and bioactivity of MMP-9 through NF-κB in human colorectal carcinoma SW480 cells.

METHODS The eukaryotic expression vector of RNA interfering (shRNA) with the Pin1 gene (pGenesil-1-PIN1) was constructed in our previous experiments and was confirmed through sequencing. Cell motility was tested through the wound healing assay and the Boyden chamber assay. The protein levels and bioactivity of MMP-9 were tested by Western blotting and gelatin zymography in SW480 cells after transfection with pGenesil-1-PIN1 (SW480/p-shRNA). The DNA-binding activity of NF-κB in cells transfected with pGenesil-1-PIN1 was analyzed by the electrophoretic mobility shift assay (EMSA). In addition, to determine whether NF-κB has direct interaction with the MMP-9 promoter derived from the genomic DNA of SW480 cells transfected with pGenesil-1-PIN1, oligonucleotides containing a putative NF-κB binding site were synthesized and EMSAs were performed.

RESULTS The results of the Boyden chamber assay showed that cell motility was reduced from 90.2 ± 6.5 per field (× 10 objective) to 49.6 ± 7.2 per field ($P < 0.05$, Student's t-test) for SW480 cells transfected with pGenesil-1-PIN1 (SW480/p-shRNA). Western blotting detected low protein levels of Pin1 and MMP-9 in SW480/p-shRNA cells. The relative protein levels of Pin1 were 0.49 ± 0.07 in SW480/p-shRNA compared with 0.94 ± 0.09 in SW480/p-Con, and MMP-9 were 0.45 ± 0.07 in SW480/p-shRNA, 0.83 ± 0.07 in SW480/p-Con ($P < 0.05$). The results of gelatin zymography showed that silencing Pin1 markedly reduced the bioactivity of MMP-9 in SW480 cells. EMSA results revealed low DNA-binding activity of NF-κB in SW480/p-shRNA cells compared to SW480/p-Con cells, and that NF-κB bound directly to the oligonucleotides which contained putative NF-κB binding sites in the MMP-9 promoter derived from the genomic DNA of SW480/p-shRNA cells.

CONCLUSION Inhibited Pin1 expression may contribute to the suppressive effect on the expression and bioactivity of MMP-9 in colon cancer SW480 cells, possibly through the transcription factor NF-κB.

KEY WORDS: colorectal cancer, Pin1, RNAi, MMP-9, NF-κB.

Introduction

In clinical practice, colorectal cancer (CRC) diagnosed in patients is often lethal when invasion and/or metastasis occur. According to the WHO, 90% of people are alive 5 years after the diagnosis of CRC in an early stage. However, once CRC has spread to nearby organs or lymph nodes, the 5-year survival rate is much lower. Only 39% of CRCs are found at an early stage. Therefore, it is important to identify the molecular pathogenesis of metastatic and invasive CRC.

Pin1, a member of the parvulin family of peptidyl-prolyl cis-trans isomerases (PPIases)[1], can act as a molecular switch by binding a subset of proteins phosphorylated at Ser/Thr-Pro motifs and regulating their biological activity through the isomerization of peptidyl-prolyl bonds[2]. Recent studies indicate that Pin1 is an important regulator of tumorigenesis and is overexpressed or underexpressed in different tumors[3-5]. It is frequently localized in nuclei and serves as a regulatory protein for a variety of transcription factors, including p53[6], cyclin E1[7], and NF-κB[8] . As Ryo et al.[8] reported, the function of NF-κB is regulated by Pin1-mediated prolyl isomerization and ubiquitin-mediated proteolysis of its p65/RelA subunit. Upon cytokine treatment, Pin1 binds to the pThr254-Pro motif in p65 and inhibits p65 binding to IκBα, resulting in increased nuclear accumulation and protein stability of p65 and enhanced NF-κB activity.

Most MMPs are regulated at the transcriptional level by several cytokines and growth factors which influence multiple signaling pathways[9]. The MMP gene promoter contains several *cis*-regulatory elements, often acting synergistically, with varying importance and effect, depending upon cell type and inducer[10]. In addition, Farina AR et al.[11] reported that MMP-9 expression was regulated transcriptionally through nuclear NF-κB (-600) elements within the MMP-9 gene and through the NF-κB element in the transcriptional upregulation of MMP-9 expression.

The purpose of this study was to investigate whether Pin1 is involved in colon cancer SW480 cell invasion by modulating MMP-9 through the transcription factor NF-κB and to provide a basis for reference for further research on CRC.

Materials and Methods

Cell culture and transfection

SW480 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin, and 100 μg/ml streptomycin and were incubated with 5% CO_2 at 37°C. Transfection of the recombinant vector was performed based on the company protocol (Invitrogen Life Technologies). Briefly, before transfection, cells were transferred to RPMI1640 medium without serum or antibiotics. Then, a mixture of vector and Lipofectamine was added to the plates. After being cultured for 6 h, cells were incubated with fresh medium for the desired time period. Twelve hours after transfection, the brightness of green fluorescence in SW480 cells was detected using fluorescence microscopy directly to evaluate the transfection efficiency.

Wound healing assay

Confluent cell monolayers were wounded by scratching with a pipette tip. Debris was removed from the culture by washing twice with PBS, and the cells were then incubated with 2% fetal bovine serum medium. Images were captured immediately after the cells were wounded, and the wound sizes were verified with an ocular ruler to ensure that all wounds were the same width at the beginning of the experiment. Wound closure was monitored microscopically 24 h after the wound was formed.

Boyden chamber assay

Migration potential of SW480 cells was measured by an *in vitro* Boyden chamber assay, which counted the number of cells crossing an 8-micron pore size PET membrane. Briefly, cells (1×10^5 for the transwell migration assay) in 0.2 ml of serum-free RPMI 1640 medium were added to the wells which had 8 μm pore membrane Boyden chambers for each. Cells were allowed to migrate for 24 h. Cells that did not penetrate the filters were removed with cotton swabs. Chambers were fixed in 100% methanol for 10 min and then stained with hematoxylin for 10 min. Finally, the chambers were rinsed in PBS and examined under a bright-field microscope. The value of migration was obtained by counting 5 fields per membrane, and the value represented the average of 3 independent experiments performed over multiple days.

Gelatin zymography

Equal amounts of proteins from conditioned medium were subjected to zymography to detect the activities of MMP-2 and MMP-9. Samples were added to each lane and subjected to 8% SDS-polyacrylamide gel electrophoresis containing 1 mg/ml gelatin (Sigma). After electrophoresis, the gel was renatured in 2.5% Triton X-100 for 30 min at room temperature and then incubated in 0.1 mol/L glycine-sodium hydroxide, pH 8.3, at 37°C for 20 h. The gel was stained with 1% Coomassie Brilliant Blue R-250 (Amersham Biosciences, Piscataway, NJ) and destained with destaining buffer (10% acetic acid and 40% methanol).

Cell lysis and Western blot analysis

Cells were washed with ice-cold PBS and lysed in a solubilization buffer (pH 8.0, 1% Triton X-100, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 100 IU aprotinin) on ice for 30 min and then centrifuged

at 13,000 × *g* for 10 min to elicit a supernatant. Protein concentration was measured by the Bradford method, and 30 μg of total protein was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel, followed by transfer to a nitrocellulose membrane. Membranes were blocked overnight with 5% nonfat milk in Tris-buffered saline (0.1% Tween-20 in TBS), and then probed, respectively, with rabbit polyclonal anti-Pin1 antibody (1:500), mouse monoclonal anti-MMP2, and mouse monoclonal anti-MMP-9 (1:500; Santa Cruz Biotech, Santa Cruz, CA) for 4 h at 4°C. After several washings, membranes were incubated with anti-rabbit or mouse IgG conjugated with horseradish peroxidase, and then washed. Subsequent analysis was performed with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Electrophoretic mobility-shift assay

EMSAs were carried out as described previously. Binding reactions were performed at room temperature for 25 min. The binding reactions contained 32P 5'-end-labeled, double-strand oligonucleotide probe, 2 μg of poly (deoxyinosinic-deoxycytidy acid), 5 μg of SW480 or SW480 nuclear extract, and additional competitor DNAs or antibody as specified in the figure legends. The oligonucleotides used in this study were as follows: commercial consensus oligonucleotide sequence of NF-κB (E3291 E3292/5'-AGT TGA GGG GAC TTT CCC AGG C-3') MMP-9-NF-κB (5'-TGC CCC AGT GGA ATT CCC CAG CCT TG-3')[11]. All oligonucleotides were purchased from Beijing Aoke Company and were double-stranded, and the complementary affinity strands were not indicated; lower case referred to mutated bases. In super shift analyses, 2 μl (2 mg/ml) of anti-p-NF-κB p65 (Thr 254) (Santa Cruz Biotech) was added to the reaction.

Statistical analysis

Student's *t*-test was used to compare the data between the 2 groups. Values were expressed as mean ± SD error of the mean. A *P*-value less than 0.05 was considered statistically significant. Statistical analysis was performed using Microsoft Office Excel software.

Results

Transfection efficiency of the Pin1 shRNA plasmid and the expression of Pin1 in SW480 cells

Forty-eight hours after transfection, we found that the transfection efficiency was about 40% in the SW480 cells (Fig.1). The cell bodies transfected with Pin1 shRNA plasmid became round and shrank, and a portion of the cells contained debris. At the same time, the number of the cells was obviously reduced and the characteristic morphology of the transfected cells changed greatly compared with that of the nontransfected cells. The expression level of Pin1 protein was then detected by Western blotting (Fig.2). The results showed that the Pin1 shRNA plasmid significantly reduced the Pin1 protein expression level compared with the negative controls and the nontransfected cells. The relative protein levels of Pin1 were 0.49 ± 0.07 in SW480/p-shRNA, 0.94 ± 0.09 in SW480/p-Con, and 1.01 ± 0.11 ($P < 0.05$, Student's *t*-test) in nontransfected SW480 cells.

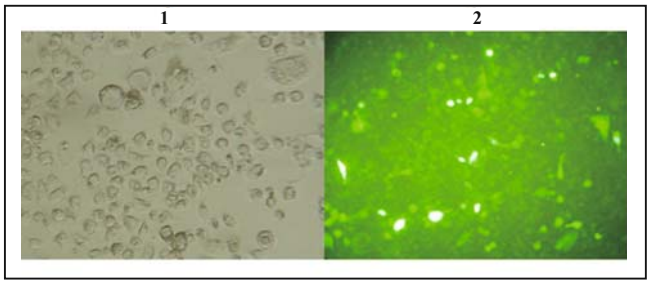

Fig.1. Fluorescent microscopy of transfected CRC cells (× 400); (1) Transfected with the Pin1 shRNA plasmid in SW480 cells by optical microscope (SW480/p-shRNA); (2) Transfected with the Pin1 shRNA plasmid in SW480 cells by fluorescent microscope (SW480/p-shRNA).

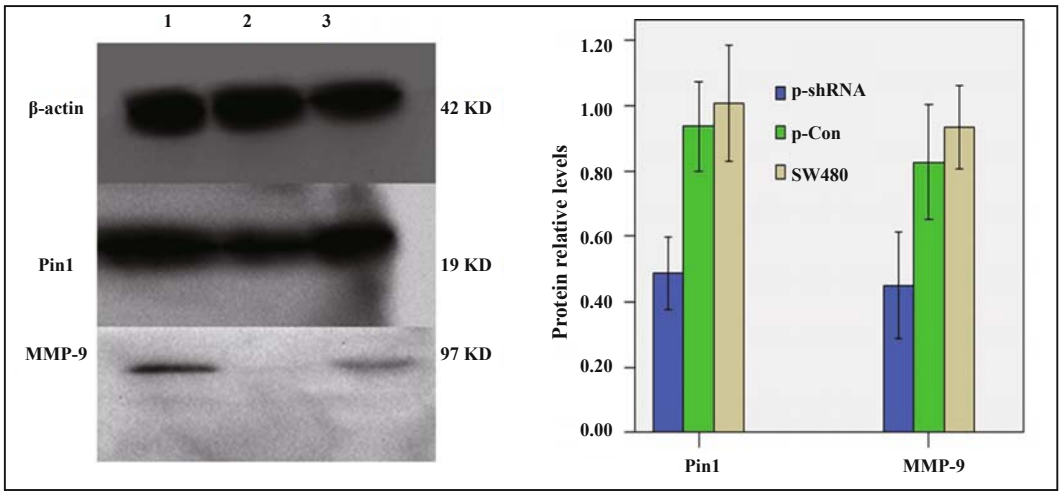

Fig.2. Western blotting of CRC cells. The reduced levels of Pin1 expression in SW480 cells 48 h after transient transfection with Pin1 shRNA plasmid, compared with controls and nontransfected cells; (1) Nontransfected SW480 cells; (2) SW480/p-shRNA; (3) SW480/p-Con.

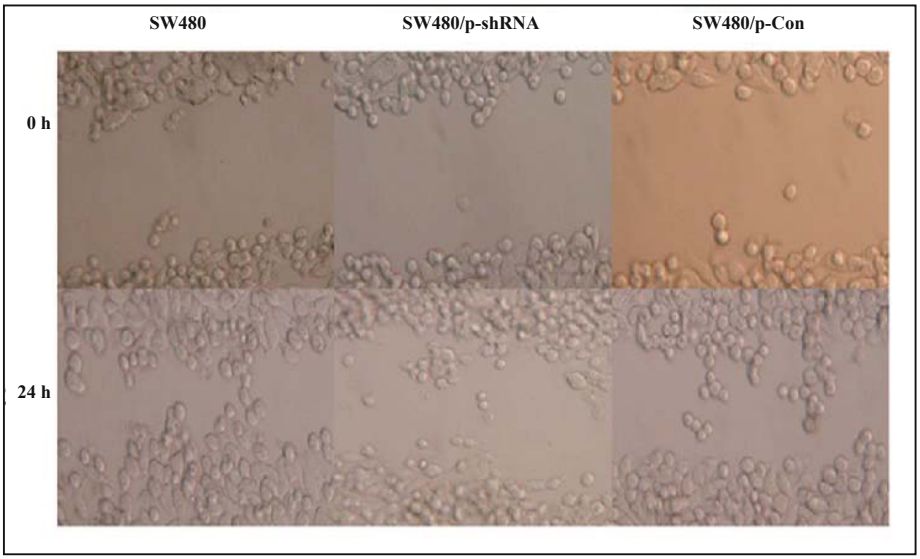

Fig.3. Wound healing assay of CRC cells. Cells were wounded by scratching with a pipette tip and the cells were incubated with RPMI-1640 medium containing no FBS for 24 h. Cells were photographed under phase-contrast microscope (× 400).

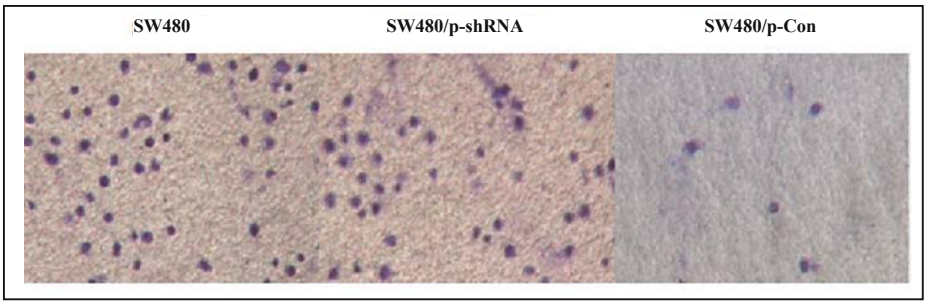

Fig.4. Boyden chamber assay in CRC cells. SW480 cells (1×10^5 cells/0.2 ml RPMI-1640) were added to the chambers and cells that migrated through the membrane to the lower surface of the filter were stained with hematoxylin and photographed under an optical microscope at × 40.

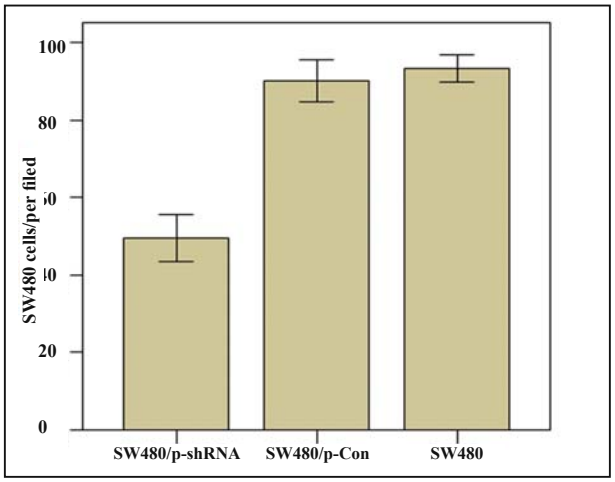

Fig.5. Migration cells/per field. The average number of cells penetrating the filters decreased from 93.3 ± 3.7 per field (× 10 objective) and 90.2 ± 6.5 per field to 49.6 ± 7.2 per field ($P < 0.05$, Student's *t*-test) in SW480/p-shRNA cells.

Silencing Pin1 inhibits the migration properties of SW480 cells

The effects of Pin1 shRNA plasmid on cell migration were investigated using the wound-healing assay. The cells in culture dishes were scraped with a plastic pipette tip to generate the same size of "wound" area. Then pictures were taken at different time points to monitor the "wound healing" ability (Fig.3). After 24 h, the cells transfected with Pin1 shRNA plasmid still had obvious "wounds", but the wounds were almost healed in the cells transfected with the controls. The results showed that SW480/p-shRNA led to greater inhibition on cell migration than SW480/p-Con.

The Boyden chamber assay was used to detect the migration properties of SW480 cells in response to the transfection of the Pin1 shRNA plasmid. After 10-hour culture in the Boyden chambers, the average number of the SW480/p-shRNA cells which migrated through the membrane was 49.6 ± 7.2 per field (× 10 objective), and that of the SW480/p-Con and the non-transfected SW480 cells were 90.2 ± 6.5 and 93.3 ± 3.7, respectively (Figs. 4, 5).

Silencing Pin1 inhibits the expression and bioactivity of MMP-9 in SW480 cells

Tumor metastasis and invasion are the most troublesome problems in the management of CRC patients. These events require diverse proteolytic enzymes, among which MMP-9 plays a significant role in the degradation of type IV collagen, the major component of the basement membrane. Levels of MMP-9 after transfection of Pin1 shRNA plasmid were examined in this study using Western blotting (Fig.2), and the activity of MMP-9 were tested by gelatin zymography (Fig.6). The protein levels of MMP-9 in SW480/p-shRNA, in SW480/p-Con, and in SW480 were 0.45 ± 0.07, 0.83 ± 0.07, and 0.93 ± 0.05, respectively.

Identification of the activity of NF-κB in SW480 cells

In order to identify whether or not NF-κB had DNA-binding activity in the SW480/p-shRNA and SW480/p- Con cells, we analyzed the DNA-binding ability in the conserved sequence of NF-κB and in SW480 nuclear extract by EMSA. The results shown in Fig.7 indicated that the binding capability of the conserved sequence of NF-κB was specific in SW480 cells, and that the binding capability in SW480/p-shRNA was weaker than in SW480/p-Con cells.

NF-κB binds directly to the oligonucleotides which contain putative NF-κB binding sites in the MMP-9 promoter (-600 elements) derived from the genomic DNA of SW480 cells

The results shown in Fig.8 demonstrated that the oligonucleotide sequence of MMP-9-NF-κB was able to specifically bind in SW480 cells and that the binding capability of SW480/p-shRNA was lower than that of SW480/p-Con cells.

Discussion

RNAi is known as post-transcriptional gene silencing (PTGS) machinery in which double-stranded RNA (dsRNA), in tandem with protein complexes, catalyzes the degradation of complementary mRNA targets. RNAi is the latest new technology to be applied in the field of genetic medicine in which specific genes can be turned off or silenced, so as to affect a therapeutic outcome. It can be highly specific, and functions in the nanomolar range, furthermore, it is far more effective than the antisense approaches popular 10-15 years ago[12,13].

Pin1 is made up of a WW interaction domain and a C-terminal catalytic subunit. Further, several high-resolution structures in Pin 1, which have helped to define its function, are available. The enzymatic activity of Pin1 towards short peptides containing the pSer/Thr-Pro motif has been well documented, and the available research on the molecular mechanisms of its isomerase activity has been reported[14]. SW480 cells transfected with p-shRNA (Pin1 shRNA plasmid) or p-Con were used to test the tumor' s metastatic and invasive potential. Both activity assays and Western blotting analysis confirmed that Pin1 was highly expressed in SW480 cells, but showed low expression in

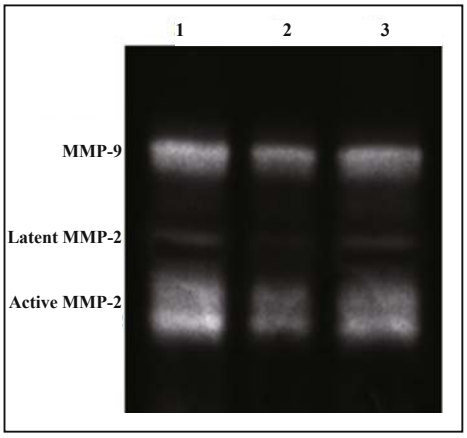

Fig.6. Gelatin zymography. (1) SW480/p-Con; (2) SW480/p-shRNA; (3) Nontransfected SW480 cells. The activity of MMP-9 in SW480/p-shRNA cells was significantly reduced in the SW480/p-shRNA and nontransfected SW480 cells.

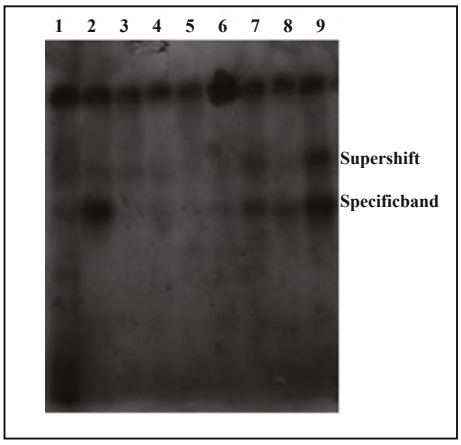

Fig.7. DNA binding activity of NF-κB in the SW480/p-shRNA and SW480/p-Con cells. Lane 1, Free probe group; Lane 2, SW480/p-Con cells group; Lane 3, SW480/p-shRNA cells group; Lane 4, 100-fold unlabelled specific probe in SW480/p-Con cells group; Lane 5, 100-fold unlabelled specific probe in SW480/p-shRNA cells group; Lane 6, 100-fold unlabelled unspecific probe in SW480/p-shRNA cells group; Lane7, Anti-NF-κB antibody in SW480/p-shRNA cells group; Lane 8, 100-fold unlabelled unspecific probe in SW480/p-Con cells group; Lane 9, Anti-NF-κB antibody in SW480/p-Con cells group.

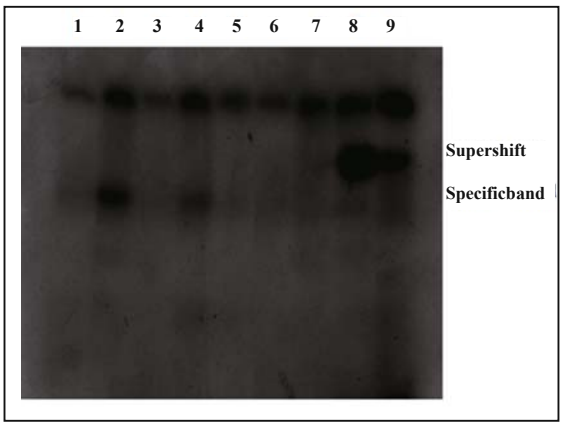

Fig.8. Specific interaction of NF-κB with the promoter region of MMP-9. Lane 1, free probe group; Lane 2, SW480/p-Con cells group; Lane 3, SW480/p-shRNA cells group; Lane 4, 100-fold unlabelled unspecific probe in SW480/p-Con cells group; Lane 5, 100-fold unlabelled unspecific probe in SW480/p-shRNA cells group; Lane 6, 100-fold unlabelled specific probe in SW480/p-Con cells group; Lane7, 100-fold unlabelled specific probe in SW480/p-shRNA cells group; Lane 8, Anti-NF-κB antibody in SW480/p-Con cells group; Lane 9, Anti-NF-κB antibody in SW480/p-shRNA cells group.

SW480/p-shRNA compared with that in SW480/p-Con cells. The results from Western blotting and gelatin zymography showed that SW480/p-shRNA cells had lower MMP-9 expression than SW480/p-Con cells. The migratory and invasive properties in SW480/p-shRNA cells were significantly decreased compared with those in SW480/p-Con cells, which were demonstrated by both the wound healing assay and the Boyden chamber assay.

Cancer cell invasion has been reported to be promoted by the activation of matrix metalloproteinases (MMPs), especially MMP-9[15-17]. MMPs belong to a family of enzymes that degrade the extracellular matrix (ECM) components and are known to contribute to tissue repair, tumor metastasis and invasion. Several reports indicated that increased levels of MMP-9 correlated with invasive properties of certain tumor cell types[18-20]. Observations from studies have shown that tumor invasion activities are related to Pin1 in CRC cells. Thus, the inhibitory effects of Pin1 shRNA plasmid on cancer cell migration and invasion lead to the hypothesis that Pin1 might affect the proteolytic activity of proteases involved in the degradation of the ECM. Therefore, the relationship between Pin1 and the major proteolytic effectors known to be involved in ECM degradation during migration and invasion were investigated.

Silencing Pin1 suppresses the binding of nuclear proteins to oligonucleotides, corresponding to the consensus sequence of NF-κB response elements in the MMP-9 promoter in SW480 cells. The super shift EMSA assay revealed that the transcription factor NF-κB (pThr254-Pro in p65) bound to the respective binding sequence in SW480 cells.

In the present study, we targeted Pin1 by RNAi and observed that Pin1 expression by RNA interference blocks then, induced MMP-9 gene expression in SW480 human CRC cells. Pin1 RNAi also suppressed the SW480 nuclear proteins binding to NF-κB response elements in the MMP-9 promoter. In conclusion, Pin1 is involved in MMP-9 expression and bioactivity in SW480 cells. The transcription factor NF-κB may be involved in the transregulation of the MMP-9 promoter by Pin1 RNAi. These results may indicate the role that the MMP-9 plays in mediating or triggering the events of invasion.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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