A functional polymorphism at miR-491-5p binding site in the 3'UTR of MMP9 gene confers increased risk for pressure ulcers after hip fracture

RUI YANG, LI HAN and XIANTIE ZENG

Department of Traumatic Ankle Surgery, Tianjin Hospital, Tianjin 300211, P.R. China

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Abstract. The roles of matrix metalloproteinase (MMP)9 in the control of pressure ulcers (PU) after hip fracture as well as how the rs1056629 in MMP9 3'UTR compromises the interaction between MMP9 and miR-491 were explored. Online miRNA database (http://www.bioguo.org) was utilized to explore gene polymorphism in MMP9 3'UTR that might break the interaction between MMP9 and miRNA. Luciferase assay was utilized to confirm the miRNA targeted MMP9. Real-time PCR, western blot analysis and immunohistochemistry were carried out to understand the roles of MMP9 in PU as well as how rs1056629 in MMP9 3'UTR compromises the interaction between MMP9 and miR-491. rs1056629 in MMP9 3'UTR that compromised the interaction between MMP9 and four miRNAs including miR-194-3p, miR-491, miR-1915-3p and miR-941, and only miR-491 among miR-194-3p, miR-491, miR-1915-3p and miR-941 decreased luciferase activity of wild-type MMP9 3'UTR, and luciferase activities of mutant-3 and mutant-4 MMP9 3'UTR in miR-491 overexpressing cells was comparable with scramble control. miR-194-3p, miR-491, miR-1915-3p and miR-941 levels in PU group was comparable with healthy control, and miR-194-3p, miR-491, miR-1915-3p and miR-941 in subjects carrying AA genotype was similar with those in AC and CC groups. MMP9 mRNA and protein, and histology score in subjects with PU were much higher, and were also much higher in AA group. Only miR-491 mimic among miR-194-3p, miR-491, miR-1915-3p and miR-941 mimics downregulated the MMP9 level, and only miR-491 inhibitor among miR-194-3p, miR-491, miR-1915-3p and miR-941 inhibitors upregulated the MMP9 level. Our study indicated that rs1056629 polymorphism could be a novel biomarker for predicting the occurrence of PU after a hip fracture.

Introduction

Pressure ulcers (PUs) are frequent problems among people who stay in hospital in chronic and acute care settings and bring a heavy pressure on patients, their families and caregivers (1). Today, PUs are one of the five leading factors of damage to patients and preventable safety condition in the world. Moreover, it is growingly considered as a marker of the quality of care offered by health care institutions (2). PUs in older inpatients can cause substantial negative impacts on medical complications, expense of care, duration of hospital stay, quality of life, pain and death (3). The main risk factor of PU is immobility; other patient features including poor nutritional status and incontinence have also been discovered to elevate the risk of PU (4). Older patients with surgical repair of a hip fracture are at a high risk because of the possibility for long-term immobility and other risk factors of PU (5).

As a common complication of hip fracture, PU has a frequency of 8.8-55%. It has substantial influence on the quality of life, hospital care cost, and death (6). Some reports investigating biopsies and wound fluids collected from PUs revealed that the healing process might be impeded by the excessive levels of active forms of matrix metalloproteinases (MMP)-2 and MMP9. These data indicate that the proteinases could destruct extracellular proteins, receptors and growth factors critical for PUs healing (7).

As endogenous ~22 nucleotide small non-coding RNAs, microRNAs (miRNAs) regulate gene production by inducing either RNA degradation or translation repression and impacting mRNAs (8). These non-coding RNAs bind to the target mRNA at the 3'UT, and the seed region of the miRNA (from nucleotide 2 to 8) mainly dictates the specificity of the binding. Many target genes, usually sharing the same pathway, are modulated by the same miRNA. A range of miRNAs can affect the same transcript to make post-transcriptional modulation more complex (9). Sequence complementarity of just seven nt located at the ‘seed region’ of miRNA (position 2 to position 8 of the miRNA) or even six nt (position 2 to position 7) with the target mRNA is generally adequate to inhibit translation though most human miRNAs consist of 22 nt on average (10). Moreover, SNPs and polymorphisms located at the 3'UTR of genes that change miRNA binding may impact the expression of protein related to the occurrence of a variety of disorders (11).
It has been previously found that rs1056629 is located within 3'UTR of MMP9, and potentially compromises the interaction between MMP9 3'UTR and miR-491, as the presence of minor allele of the SNP breaks the binding site of the miRNA (12). rs1056629 has been reported to be associated with an elevated risk of coronary heart disease (12). Simultaneously, MMP9 has been shown to be an important enzyme involved in the development of pressure ulcer (13,14). In this study, we studied the association between the rs1056629 and the risk of pressure ulcer in the patients with hip fracture, as well as its effect on the serum level of MMP9.

Materials and methods

Samples. Forty individuals comprising 22 subjects suffering from PU and 18 healthy controls, were recruited for this study. Additionally, the 22 subjects with PU were diagnosed just after having hip fracture in our hospital. Tissue samples were collected from all subjects, and stored in -80°C for further analysis. Participants aged >90 years, malignant origin, psychiatric and obstetric patients, allergy to wound products were excluded from our study. All procedures and use of tissue samples were approved by the Ethics Committee of our institute, and participants or their first-degree relatives had already signed the informed consents before start of the experiment after all potential risk factors were carefully explained. The study was conducted according to the Declaration of Helsinki.

Genotyping by TaqMan. DNA extraction kit (Qiagen, Dusseldorf, Germany) was used to extract the DNA from peripheral blood samples in accordance with the manufacturer's protocol. PCR was used to amplify the DNA fragments including rs1056629. The PCR products were sequenced in forward direction, and TaqMan genotyping kit (Qiagen) and BLAST were used to determine the SNP genotypes. Each experiment was performed three times.

In silico analysis. We search public online tool (http://www.bioguo.org) to find gene polymorphism in MMP9 3'UTR that might break the interaction between MMP9 and miRNA.

RNA isolation and real-time PCR. TRILzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract total RNA from BJ fibroblast cells and tissue samples according to the manufacturer's instructions. M-MLV (Invitrogen) was utilized to synthesize the first stranded cDNA from 2 µg total RNA in a 25 µl mixture following guideline suggested by vendor. Agarose gels (2%) with 5 mg/ml nucleic acid dye was utilized to electrophorese the PCR product, and AlphaEaseFC software (Genetic Technologies, Miami, FL, USA) was utilized to conduct expression analysis of MMP9. An iCycler iQ real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR Green Master Mix (QPK-201; Toyobo, Co., Ltd., Osaka, Japan) was utilized to perform real-time PCR, and the reaction was carried out at 95°C for 5 min, 45 cycles of (25 sec at 95°C; 20 sec at 60°C; 30 sec at 72°C). U6 RNA and GAPDH served as internal controls. 2-ΔΔCt method was utilized to analyze the relative expression of miR-194-3p, miR-491, miR-1915-3p, miR-941 and MMP9 mRNA. Three independent experiments were repeated.

Cell culture and transfection. BJ fibroblast cells were obtained from Lonza (Basel, Switzerland), and RPMI-1640 medium (Gibco) containing 10% FBS (fetal bovine serum) (Sijjing, Hangzhou, China) and 1% penicillin-streptomycin was utilized to culture these cells under a humid atmosphere with 5% CO2/95% air at 37°C. Prior to transfection, cell were seeded into 96-well plates at a final density of 1x10^5 per well, when the cells grew to 80% confluence, they were co-transfected with miR-194-3p mimics/inhibitors, miR-491 mimics/inhibitors, miR-1915-3p mimics/inhibitors, miR-941 mimics/inhibitors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three independent experiments were repeated.

Vector construction and mutagenesis. PCR was performed to amplify fragment of MMP9 cDNA, and then PCR products were inserted into pGL3 vector (Promega Corp., Madison, WI, USA), then direct sequencing was adopted to confirm the accuracy. The QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies) was used to perform the single-base mutation in gene polymorphism located in binding site of miR-194-3p (A allele to C allele), miR-491 (A allele to C allele), miR-1915-3p (G allele to A allele) or miR-941 (G allele to A allele) in MMP9 3'UTR according to the manufacturer's instructions, and meanwhile QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies) with carefully designed primers was used to perform the miR-194-3p, miR-491, miR-1915-3p or miR-941 seed sequence mutation in MMP9 3'UTR. The above eight mutagenesis were performed for the same site and introduced to the pGL3 vector (Promega Corp.) at the same time, direct sequencing was adopted to confirm the mutation site accuracy. Three independent experiments were performed.

Luciferase assay. Full length of MMP9 3'UTR containing predicted binding site of miR-194-3p, miR-491, miR-1915-3p or miR-941 was amplified using PCR, then the above PCR products were inserted into pGL3-control vector (Promega Corp.), and located downstream of the luciferase gene, and obtained pGL3-MMP9-3'UTR construct. Then the ‘seed sequence’ in MMP9 3'UTR was mutated, and PCR was also performed to obtain point mutations, and cloned into pGL3-control vector (Promega Corp.) to generate pGL3-MMP9 3'UTR Mut construct. 1x10^3 BJ fibroblast cells were seeded into 96-well plates for 24 h. Lipofectamine 3000 (Invitrogen) was utilized to transfect miR-194-3p, miR-491, miR-1915-3p or miR-941 mimic or NC (negative control) RNA oligonucleotide and 0.4 mg of pGL3 constructs along with 0.07 mg of pRL-CMV plasmid-expressing renilla luciferase into BJ fibroblast cells. Dual Luciferase Reporter assay system (Promega Corp.) was utilized to detect luciferase activity 48 h post-transfection, and luciferase activity of firefly was normalized to that of renilla luciferase. Three independent tests were repeated.

Western blot analysis. The total protein was extracted from BJ fibroblast cells and tissue samples using lysis buffer (Beyotime, Haimen, China), and Enhanced BCA Protein assay kit (Byotime) was utilized to measure protein
Polyacrylamide gel (10%) (w/v) with SDS-PAGE was utilized to separate protein extracts, and then electro-transferred onto PVDF (polyvinylidene difluoride) (PerkinElmer, Waltham, MA, USA) membrane for 90 min. Non-fat milk (5%) was utilized to block the membrane. Then monoclonal antibodies against anti-human MMP9 (1:5,000; Abcam, Cambridge, MA, USA) at 1:12,000 dilution β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were utilized to incubate the membranes for 12 h at 4°C, next, HRP (horse radish peroxidase)-linked mouse anti-goat secondary antibody at 1:15,000 dilution (1:3,000, Santa Cruz Biotechnology, Inc.) was utilized to treat the membranes for another 1 h. The Fusion FX7 system (VilberLourmat, Marne-la-Vallée, France) with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to visualize the bands, and ImageJ Analyst software [National Institutes of Health (NIH), Bethesda, MD, USA] was utilized to quantify the level of MMP9. Three independent experiments were carried out.

Immunohistochemistry. Anti-MMP9 antibody (1:500; Abcam) was utilized to carry out immunohistochemistry for MMP9 on a 4-µm tissue sample. Xylene was utilized to bake and deparaffinize the slides, and graded alcohol was utilized to pass through the above slides, next 1 mM EDTA (Invitrogen) was utilized to retrieve antigen in a steam pressure cooker (Decloaking Chamber; BioCare Medical, Walnut Creek, CA, USA) for 30 sec at 125°C. The following experiments were performed in a hydrated chamber at room temperature. Peroxidase Block (Dako, Carpentaria, CA, USA) was utilized to pretreat the slides for 5 min to quench the activity of endogenous peroxidase, and 50 mM Tris-Cl, pH 7.4 was utilized to wash the slides, 5 ml 50 mM Tris-Cl containing 250 µl normal goat serum (Dako) was utilized to block the slides, anti-MMP9 antibody (1:500; Abcam) to incubate the slides for 60 min. Tris-Cl (50 mM), pH 7.4 was utilized to wash the slides, rabbit HRP conjugated Signal stain boost IHC detection reagent (Cell Signaling Technology, Inc., Danvers, MA, USA) was utilized to treat the slides for 30 min, next, DAB (3,3'diaminobenzidine) chromogen (Dako) was utilized to develop immunoperoxidase staining for 5 min, followed by counterstaining with hematoxylin. Following staining, two experienced pathologists scored immunostaining independently, two pathologists were blinded to the clinical outcomes and clinicopathological parameters of the subjects. The scores of the two pathologists with any discrepancy were re-examining by both pathologists to obtain a consensus score.

Statistical analysis. The data are presented as means ± SD (standard deviation). GraphPad Prism 5.0 (GraphPad Software Inc. La Jolla, CA, USA) was utilized to perform the statistical analyses. Unpaired t-test was utilized for comparison of the result of real-time PCR. Chi-square test was employed to analyze qualitative data, and Pearson correlation analysis to determine the correlation. P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-491 directly targets MMP9. We searched online miRNA database (http://www.bioguo.org) to identify the gene polymorphisms in MMP9 3'UTR that might potentially interfere with the interaction between MMP9 and miRNA, and found that gene polymorphism MMP9 3'UTR might compromise the interaction between MMP9 with miR-194-3p, miR-491, miR-1915-3p or miR-941, and QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies, CA, USA) was used to introduce the mutations (either the minor allele of the target polymorphism or the complementary sequence of the seed sequence) located in binding site of miR-194-3p (mutant-1 and mutant-2) (Fig. 1A), miR-491 (mutant-3 and mutant-4), miR-1915-3p (mutant-5 and mutant-6), miR-941 (mutant-7 and mutant-8), respectively.

![Figure 1](image-url)
YANG et al: rs1056629 CONFFERS INCREASED RISK FOR PRESSURE ULCERS

Luciferase assay was performed to test whether miR-194-3p, miR-491, miR-1915-3p or miR-941 directly regulated MMP9 expression or the effect of the candidate polymorphism on the interaction between the miRNAs and MMP9. The cells were co-transfected with constructs containing wild-type MMP9 3'UTR and mutant-1-9 MMP9 3'UTR and miR-194-3p, miR-491, miR-1915-3p or miR-941 mimic. As shown in Fig. 2, only transfection with miR-491 (Fig. 2B) significantly reduced luciferase activity of wild-type MMP9 3'UTR, whereas luciferase activity of mutant-3 and mutant-4 MMP9 3'UTR showed no obvious difference in comparison with scramble control. By contrast, luciferase activity of co-transfected constructs containing wild-type MMP9 3'UTR and mutant-1/2/5/6/7/8 MMP9 3'UTR and miR-194-3p, miR-491-3p or miR-941 mimic was comparable with scramble control, respectively. The results indicated that miR-491 directly targeted MMP9, and rs1056629 disrupted the interaction between MMP9 and miR-491.

Differential expression of miR-194-3p, miR-491, miR-1915-3p or miR-941 and MMP9 in different groups. Forty individuals comprising with (N=22) or without (N=18) PU were recruited for this study, and all those subjects were rs1056629 genotyped. Real-time PCR was performed to detect expressions of miR-194-3p, miR-491, miR-1915-3p or miR-941 and MMP9 in these subjects. As shown in Fig. 3, miR-194-3p (Fig. 3A), miR-491 (Fig. 3C), miR-1915-3p (Fig. 3E) and miR-941 (Fig. 3G) levels in patients with PU was similar with the controls, and miR-194-3p (Fig. 3B), miR-491 (Fig. 3D), miR-1915-3p (Fig. 3F) and miR-941 (Fig. 3H) levels are comparable with each other among AA, AC and CC genotype groups. MMP9 mRNA level in patients with PU (Fig. 4A) was much higher than healthy controls, and also much higher in participants carrying AA genotype (Fig. 4B) than AC and CC genotypes, suggesting that interaction between miR-491 and MMP9 could be disrupted by the presence of the minor allele of the rs1056629 polymorphism.

Differential protein levels of MMP9 and histology score in different groups. Immunohistochemistry was carried out to determine MMP9 protein level among AA, AC and CC groups. As shown in Fig. 5A-C, MMP9 protein was highly expressed in AA group compared to that in AC and CC group, furthermore histology score in PU group was much higher in PU group than control group (Fig. 5D), and was also much higher in AA group than AC and CC groups (Fig. 5E), and histology score in AC and CC was similar with each other.

Figure 2. (A) miR-194-3p had no effect on luciferase activity of wild-type, mutant-1 and mutant-2 MMP9 3'UTR. (B) miR-491 decreased luciferase activity of wild-type MMP9 3'UTR, but not those of mutant-3 and mutant-3 MMP9 3'UTR. (C) Luciferase activities of wild-type, mutant-5 and mutant-6 MMP9 3'UTR were similar with scramble control. (D) Luciferase activities of wild-type, mutant-7 and mutant-7 MMP9 3'UTR showed no obvious difference with scramble control.
Figure 3. Differential expression of miR-194-3p, miR-491, miR-1915-3p or miR-941 in different groups. (A) miR-194-3p in PU group was similar with control. (B) miR-194-3p in subjects carrying AA genotype was similar with healthy controls. (C) miR-491 in PU group was comparable with control. (D) miR-491 in participants carrying AA genotype was comparable with healthy controls. (E) miR-1915-3p level in PU group showed no significant difference with control group. (F) miR-1915-3p level in AA group showed no significant difference with AC and CC group. (G) miR-941 in PU group was comparable with control. (H) miR-941 in participants carrying AA genotype was comparable with healthy controls.

Figure 4. Differential expression of MMP9 mRNA in different groups. (A) MMP9 level in subjects with PU was much higher than healthy controls. (B) MMP9 level in subjects carrying AA genotype was much higher than AC and CC group.
Effect of up- or downregulation of four miRNAs on the expression of MMP9. Real-time PCR and western blot analysis were performed to explore the effect of up- or downregulation of four miRNAs on the expression of MMP9. MMP9 levels in BJ fibroblast cells transfected with miR-194-3p, miR-491, miR-1915-3p or miR-941 mimic/inhibitor were measured. As shown in Fig. 6, only cells transfected with miR-491 mimic (Fig. 6B) showed evident decrease in MMP9 mRNA and protein compared with scramble control, while MMP9 mRNA and protein in BJ fibroblast cells transfected with miR-194-3p (Fig. 6A), miR-1915-3p...
Discussion

The loss of miR-491, has been identified in several cancers, including breast cancer (colorectal cancer, glioblastoma and hepatocellular carcinoma (HCC) (15-18), miR-491-5p, a mature form of miR-491, has been demonstrated to inhibit the growth and metastasis of cervical cancer, breast cancer, pancreatic cancer and ovarian cancer by impacting hTERT genes, JMJD2B, TP53 and Bcl-XL, respectively (19). However, a report indicated that the level of miR-495-5p was elevated in colon cancer, particularly in those aged ≥70 years, and high miR-491-5p expression related to poor overall survival of patients with colon cancer indicating that miR-491-5p acts as oncogene in colon cancer (20). miR-491 promotes cell apoptosis by impacting Bcl-XL, and hence inhibits the proliferation of colorectal cancer cells (17). Moreover, in pancreatic cancer, miR-491 could act on TP53 and Bcl-XL to trigger cell apoptosis via a pathway induced by mitochondria (21). In addition, miR-491 impacted a range of oncogenes, such as EGFR, Wnt signal and GIT-1 to weaken the malignant features (16). Moreover, miR-491 functioned as an anti-metastasis gene. G-protein-coupled receptor kinase-interacting protein 1 and MMP9 could be targets of miR-491 (22). In this study, we identified that MMP9 is a direct target of miR-491 by showing that only cells transfected with miR-491 mimic showed evident decrease in MMP9 mRNA and protein, and only cells transfected with miR-491 inhibitor showed evident increase in MMP9 mRNA and protein compared with scramble control.

MMP-induced proteolysis is critical in regulation of cellular homeostasis: MMPs can start, enhance, or reduce signaling homeostasis: MMPs can start, enhance, or reduce signaling and modify tissue architecture (23). MMP9 (known as gelatinase B) is one of the 23 MMP family members and exhibits promise as a therapeutic target, based on a great deal of data showing its involvement in pathological processes accounting for tumorigenesis, metastasis and chronic inflammation (24). Reduced MMP9 expression and activity are related to a range of inflammatory problems, such as ulcerative colitis (UC) (23,25). In areas of active disease, UC is a recurrent/remitting autoimmune colonic inflammation characterized by mediation of proteolytic activity and protein levels of MMP9 (26). MMP9 activity in UC is involved in both perpetuation and generation of an inflammatory state medicated by pro-inflammatory cytokines including IL1-α and TNF-α and it can assist to maintain pro-inflammatory processes by activating IL1-β, by potentiating IL-8, and by liberating TGF-α and TNF-α (27,28). MMP9 also accounts for the inflammatory milieu via proteolysis of the basement membrane (BM) compositions laminin and collagen IV (29). Epithelial cell apoptosis can be caused by damage of epithelial BM, a defined character of UC, which results in the loss of integrity of the colonic mucosal epithelial barrier, thus worsening inflammation (26,30). Likely, damage of the endothelial BM can promote lymphocyte and neutrophil migration to the inflammatory site (31).

Intriguingly, a range of studies have reported the activity and production of MMP9 in various pathological and physiological processes (32). It starts to impede wound closure, suppresses cell migration and degrades the extracellular matrix in the bed skin in large quantities, and for long periods in the incorrect site (33). However, Gumieiro et al showed that pro-MMP9 was related to gait status recovery six months following hip fracture but not related to PU and mortality in those with hip fracture (13). Some analysis of fluids and wound biopsies obtained from patients with PU indicated increased expression of the activated MMP9. This evidence indicated that this protease could destruct the extracellular proteins, receptors and growth factors critical for PU healing (7). It has been shown that the activity of serum pro-MMP9 was elevated more in those with PU than the healthy people (34).

In this study, we searched online miRNA database (http://www.bioguo.org), and found rs1056629 in MMP9 3’UTR that might break the interaction between MMP9 with miR-491-3p, miR-491-5p or miR-941. We utilized site-directed mutagenesis kit to obtain the 4 single-base mutations in gene polymorphism and 4 seed sequence mutations located in binding site of above 4 miRNAs in MMP9 3’UTR, and conducted luciferase assay, then revealed that miR-491 directly targeted MMP9, and rs1056629 broke the interaction between MMP9 and miR-491. Furthermore, we enrolled 40 individuals comprising 22 subjects with PU and 18 controls, and found that 4 miRNAs in patients with and without PU, or in AA, AC and CC were comparable with each other. And MMP9 mRNA in PU group was much higher than control, and was also highly expressed in AA group. In addition, we investigated MMP9 protein level among AA, AC and CC groups using immunohistochemistry, and found that MMP9 protein was highly expressed in AA group, and histology score in PU group was much higher in PU group than control group, and was also much higher in AA group than AC, CC groups. rs1056629 polymorphism is situated in the 3’UTR of MMP9 and it can assist to maintain pro-inflammatory processes accounting for tumorigenesis, metastasis and chronic inflammation (24). Reduced MMP9 expression and activity are related to a range of inflammatory problems, such as ulcerative colitis (UC) (23,25). In areas of active disease, UC is a recurrent/remitting autoimmune colonic inflammation characterized by mediation of proteolytic activity and protein levels of MMP9 (26). MMP9 activity in UC is involved in both perpetuation and generation of an inflammatory state medicated by pro-inflammatory cytokines including IL1-α and TNF-α and it can assist to maintain pro-inflammatory processes by activating IL1-β, by potentiating IL-8, and by liberating TGF-α and TNF-α (27,28). MMP9 also accounts for the inflammatory milieu via proteolysis of the basement membrane (BM) compositions laminin and collagen IV (29).
References


