A functional polymorphism at the miR-491-5p binding site in the 3'-untranslated region of the MMP-9 gene increases the risk of developing ventilator-associated pneumonia

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Abstract. Matrix metalloproteinase (MMP)-9 is associated with the severity of ventilator-associated pneumonia (VAP), while an rs1056629 SNP located in the 3'-untranslated region (UTR) of MMP-9 affects the microRNA (miRNA/miR)-491-mediated regulation of MMP-9 expression. In the present study, the effect of rs1056629 on the development of VAP in patients with chronic obstructive pulmonary disease (COPD) was investigated. Patients with COPD were enrolled in the study and their genotypes of rs1056629 (CC, CA or AA) were determined. ELISA was used to analyze the levels of TNF-α and IL-6 in the monocytes of patients with COPD carrying differential genotypes of rs1056629. Reverse transcription-quantitative PCR was carried out to evaluate the expression of miR-491 and MMP-9 mRNA in the different groups of patients with COPD. Luciferase assay was used to confirm the inhibitory role of miR-491 in MMP-9 expression. Western blot analysis was carried out to assess the expression of MMP-9 protein in A549 and H1299 cells transfected with miR-491 mimics. The risk and severity of VAP were significantly elevated in patients with COPD carrying the CC and AC genotypes of rs1056629. Although there was no difference in the expression of miR-491 in patients carrying different genotypes of rs1056629, the expression levels of TNF-α, IL-6 and MMP-9 were increased in patients with COPD carrying the CC and AC genotypes of rs1056629. The results of luciferase assay revealed that miR-491 inhibited the expression of MMP-9 through direct binding to the 3'UTR of MMP-9. Transfection of miR-491 mimics into A549 and H1299 cells markedly suppressed the expression of MMP-9 in a concentration-dependent manner. On the whole, the findings of the present study confirm that the CC and AC genotypes of rs1056629 increase the risk of developing VAP in patients with COPD by increasing the expression of MMP-9.

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Abbreviations: VAP, ventilator-associated pneumonia; CPIS, clinical pulmonary infection score; PBMCs, peripheral blood monocytes

Key words: ventilator-associated pneumonia, MMP-9, single nucleotide polymorphism, microRNA

Introduction

As a primary contributor of sepsis in elderly patients, ventilator-associated pneumonia (VAP) affects up to 30% patients in intensive care units (ICUs) with a mortality rate of ~60% (1,2). Although VAP seems to be triggered by the intubation and related mechanical ventilation procedures applied to patients in the ICU, that promote the exposure of these patients to various types of pathogens that are resistant to ordinary antibiotics, the detailed mechanisms underlying the pathogenesis of VAP remain unclear (3). In particular, since the genetic conditions of patients can be affected by a wide range of single nucleotide polymorphisms (SNPs) in their genes, their defense and immunity against pathogens, such as viruses, bacteria, fungi and other harmful microorganisms, can be significantly affected due to their differential expression of various cytokines, receptors and pro-inflammatory factors (3).

As a type of short RNA transcript of ~22 nucleotides in length with no protein encoding abilities, microRNAs (miRNAs or miRs) can regulate the expression of their target genes at the post-transcriptional level (4). miRNAs are involved functionally in the regulation of a wide range of cellular processes, such as the apoptosis, invasion, differentiation,

growth and proliferation of different types of cells (5). In addition, miRNAs can act as either tumor suppressors or oncogenes to affect the onset, development, prognosis and metastasis of a wide range of malignant tumors (3,6).

A number of miRNAs can interact with the same mRNA transcript to play a gene regulatory role at the post-transcriptional level through a complex network of signaling pathways (7). For example, SNPs positioned in the 3'-untranslated regions (UTRs) of target genes of miRNAs can alter the binding affinity between these target mRNAs and their targeting miRNAs, resulting in the differential expression of these target genes (8). In particular, the rs1056628 SNP found in the seed sequence of miR-491 may affect the expression of one of its targets, matrix metalloproteinase (MMP)-9, due to the complementary binding between miR-491 and the MMP-9 3'UTR (9). Moreover, the A/C variants of rs1056628 SNP located in the MMP-9 3'UTR have been shown to increase the incidence of atherosclerotic cerebral infarction (ACI) in Chinese patients; in addition, it demonstrated that a significant association existed between the risk of ACI and a haplotype of MMP-9, i.e., the combination of three SNPs of rs9509T, rs1056628C and rs20544C (10).

The concentration, as well as the activity of MMP-9 in the plasma of patients with VAP have been shown to be markedly increased as compared with a control group of healthy patients free of VAP (11). Thus, the plasma level of MMP-9 protein in patients with chronic obstructive pulmonary disease (COPD) may be used as a potential biomarker to determine the necessity of antibiotic treatments provided to decrease the chance of VAP (11). As a member of the superfamily of zinc-dependent endopeptidases, the 92-kDa MMP-9, which is also a member of type IV collagenase, can play a vital role in the initialization of immune responses (12-14). MMP-9 can be generated by a wide range of cells, such as monocytes, leukocytes, macrophages, keratinocytes, as well as malignant tumor cells (15). In addition, MMP-9 can play an essential role in inflammation by promoting the synthesis and release of reactive oxygen species from neutrophils (16).

It has been found that the plasma matrix MMP-9 level is associated with the severity of VAP (11). The rs1056629 SNP situated at the 3'UTR of MMP-9 has been found to increase the expression of MMP-9 by interrupting the interaction between MMP-9 and miR-491 (9). In the present study, patients with COPD who developed VAP were enrolled and the effects of rs1056629 on the expression of MMP-9 and the severity of VAP were examined.

Materials and methods

Patients and sample collection. Peripheral blood samples were collected from a total of 96 patients with COPD hospitalized at the ICU of Qinghai Red Cross Hospital from September, 2015 to August, 2017 for clinical analysis. Although all patients treated with mechanical ventilation were eligible for the screening of the study, all enrolled patients must have experienced at least one episode of VAP. Peripheral blood samples were collected from all subjects to isolate their monocytes and to determine their genotypes of rs1056629 SNP. The isolation process was accomplished using the Human Peripheral Blood Mononuclear Cell Isolation and Viability kit (ab234628; Abcam) following the instructions of the manufacturer. Subsequently, based on the results of rs1056629 SNP genotyping, all the subjects were divided into 3 groups as follows: The CC group (n=18), the CA group (n=33) and the AA group (n=45). The protocol of the study, as well as the template of informed consent form (ICF) was reviewed and approved by the Clinical Ethics Committee of our Qinghai Red Cross Hospital for the retrospective use of these blood samples, and written informed consent was obtained from all subjects or their family members prior to the initialization of the study.

Clinical pulmonary infection score (CPIS) evaluation. The CPIS of each subject was assessed using an established method as described in a previous study (17).

Genotyping by TaqMan assay. First, peripheral blood samples were collected under fasting conditions from each subject using EDTA blood collection tubes to isolate mononuclear cells. The genomic DNA in each sample of mononuclear cells was isolated from archived pellets utilizing a QIAamp genomic DNA extraction assay kit (Qiagen, Inc.) following the instructions provided with the assay kit. The genotypes of rs1056629 SNP in the genomic DNA isolated from the mononuclear cells of each subject were determined using quantitative PCR (qPCR), which was carried out using a TaqMan genotyping assay kit (cat. no. 4381657; Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 7900HT fast real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) following a standard protocol provided by the manufacturer. For the purpose of quality control, both negative control wells, which contained blank samples free of DNA, and positive control wells, which contained genomic DNA samples of a known genotype of rs1056629 SNP, were set up in each microtiter plate of the qPCR reaction.

RNA isolation and reverse transcription-qPCR (RT-qPCR). Peripheral blood samples collected from each subject and the A549 and H1299 cells (described below) were subjected to treatment with a mirVana assay kit (Ambion; Thermo Fisher Scientific, Inc.) following the instructions provided by the kit manufacturer to collect and purify the total RNA content in each sample. Subsequently, the integrity and concentration of each purified RNA sample were evaluated utilizing an Agilent Bioanalyzer (Model 2100, Agilent Technologies, Inc.). Reverse transcription was then performed utilizing a MiScript Reverse Transcription kit (cat. no. 218160; Qiagen GmbH) to produce cDNA templates, which were then subjected to qPCR utilizing specific TaqMan probes and Universal TaqMan Master Mix (cat. no. 4304437; Applied Biosystems; Thermo Fisher Scientific, Inc.) in accordance with the instructions provided by the manufacturer. All qPCR reactions were carried out in triplicate wells of a 384-well qPCR plate, which was then loaded into a 7900HT fast real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) for operation. The thermocycling conditions were 95˚C for 15 min (initial activation), 94˚C for 15 sec (denaturation), 55˚C for 30 sec (annealing), and 72˚C for 60 sec (extension). Finally, the relative expression of miR-491 and MMP-9 mRNA in each sample was calculated by normalization vs. the expression of the U6 (for miR-491) and GAPDH (for MMP-9 mRNA) housekeeping genes using the

$2^{-\Delta\Delta Cq}$ method, respectively (18). The primers used for PCR were as follows: miR-491 forward, 5'-AGTGGGGAACCCTTCC-3' and reverse, 5'-GAACATGTCTGCGTATCTC-3'; MMP-9 forward, 5'-GCCACTACTGTGCCTTTGAGTC-3' and reverse, 5'-CCCTCAGAGAATCGCCAGTACT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; and GAPDH forward, 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Cell culture and transfection. The human lung adenocarcinoma cell line, A549 (cat. no. CRM-CCL-185™), and the human lung carcinoma cell line, H1299 (cat. no. CRL-5803), were obtained from the American Type Culture Collection and incubated in a Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 2 mM L-glutamine, 10% heat inactivated FBS and 100 U/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The culture conditions were 95% air, 5% CO_2, saturated humidity and a temperature of 37˚C. These cell lines were selected due to the fact that they exhibited good growth conditions and were easier to obtain during the study. When the cells reached the logarithmic growth, they were divided into 3 groups as follows: The NC group, the 50 nM miR-491 mimics (Thermo Fisher Scientific, Inc.) group and the 100 nM miR-491 mimics (Thermo Fisher Scientific, Inc.) group, and transfected with either a scramble miRNA control sequence (5'-UGGGCGUAUAGACGUGUUACAC-3') or the corresponding concentrations of miR-491 mimics (Thermo Fisher Scientific, Inc.) using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C for 48 h following a standard transfection protocol provided by the manufacturer. Subsequent observations were performed at 48 h post-transfection.

Vector construction and luciferase assay. To determine the regulatory association between miR-491 and MMP-9, as well as the effect of different genotypes of rs1056629 SNP on the binding affinity between miR-491 and MMP-9 3'UTR, the 3'UTR of MMP-9 containing the rs1056629-A or rs1056629-C allele in its miR-491 binding site were respectively sub-cloned into pcDNA luciferase vectors (Promega Corporation). The A549 and H1299 cells were then co-transfected with different vectors of MMP-9 3'UTR in conjunction with 20 pmol miR-491 mimics or a scramble control for 24 h at 4˚C using Lipofectamine 3000®, followed by the detection of luciferase activity of the transfected cells at 48 h following the start of transfection using a Bright-Glo luciferase assay kit (Promega Corporation) following the protocol provided with the kit. The relative luciferase activity was normalized to the *Renilla* luciferase activity.

Western blot analysis. The collected clinical samples, as well as the cultured cell samples were first lysed in a RIPA lysis buffer (pH 7; Cell Signaling Technology, Inc.) containing 0.5% sodium deoxycolate, 10 mM EDTA, 0.5% NP-40, 100 mM NaCl, 100 mM Tris, and a cocktail of phosphatase and protease inhibitors (Cell Signaling Technology, Inc.). The supernatant of each sample was then collected via 30 min of centrifugation at 4˚C and 14,000 x g, followed by the quantification of the protein concentration using a BCA assay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, the protein was resolved using 10% SDS-PAGE and transferred onto a PVDF membrane, which was then blocked with 5% skim milk, incubated at 4˚C for 12 h with primary anti-MMP-9 antibody (1:1,000; ab38898; Abcam) and subsequently incubated at room temperature for 1 h with corresponding HRP-labeled secondary antibody (1:2,000; ab6721; Abcam) consecutively, developed using an enhanced chemiluminescence reagent (Amersham; Cytiva), visualized using a Bio-Rad imager (Bio-Rad Laboratories, Inc.) and processed using ImageJ software (V1.4.1; National Institutes of Health) to determine the relative expression of MMP-9 proteins utilizing β-actin as the internal reference.

ELISA. The levels of TNF-α and IL-6 in monocytes isolated from the peripheral blood samples of all subjects were determined using commercial ELISA kits (E-EL-H0109 for TNF-α, E-EL-H0102 for IL-6, Elabscience) following the kit manuals, and the absorbance values were measured utilizing a Multiskan GO microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis. All statistical analyses were carried out utilizing SPSS 16.0 statistical software (SPSS, Inc.). Continuous parameters are presented as the mean ± SD, and inter-group comparisons were carried out using one-way ANOVA with the Student-Newman-Keuls post hoc test. All statistical tests were bilateral and P-values <0.05 were considered to indicate statistically significant differences.

Results

Patients with COPD with the AC and CC genotypes of rs1056629 have a higher risk of developing VAP. All 96 patients with COPD in the present study were genotyped for their rs1056629 SNP, which was located within the 3'UTR of MMP-9 mRNA. As shown in Fig. 1, carriers of either one or two C alleles had a significantly shorter time to develop VAP when compared to the carriers of the wild-type AA (Fig. 1A). Consistently, the CPIS was notably increased in both the CC and CA groups (Fig. 1B).

Genotypes of rs1056629 SNP are associated with the differential expression of TNF-α, IL-6 and MMP-9. ELISA was carried out to evaluate the expression of TNF-α and IL-6 in the monocytes of the 3 groups of patients. The expression of TNF-α and IL-6 was evidently elevated in patients with the CC and AC genotypes than in those with the AA genotype (Fig. 2). Furthermore, the expression of miR-491 and MMP-9 mRNA was also analyzed using RT-qPCR, and no marked differences in the expression of miR-491 were found between all 3 groups (Fig. 3A). However, the expression of MMP-9 mRNA was significantly increased in the CC and AC groups (Fig. 3B).

miR-491 inhibits the expression of MMP-9 by directly binding to the 3'UTR of MMP-9. The screening of potential binding sites of miR-491 identified a miR-491 binding site at the 3'UTR of MMP-9 mRNA. Subsequently, luciferase reporter plasmids of MMP-9 3'UTR containing different genotypes of rs1056629

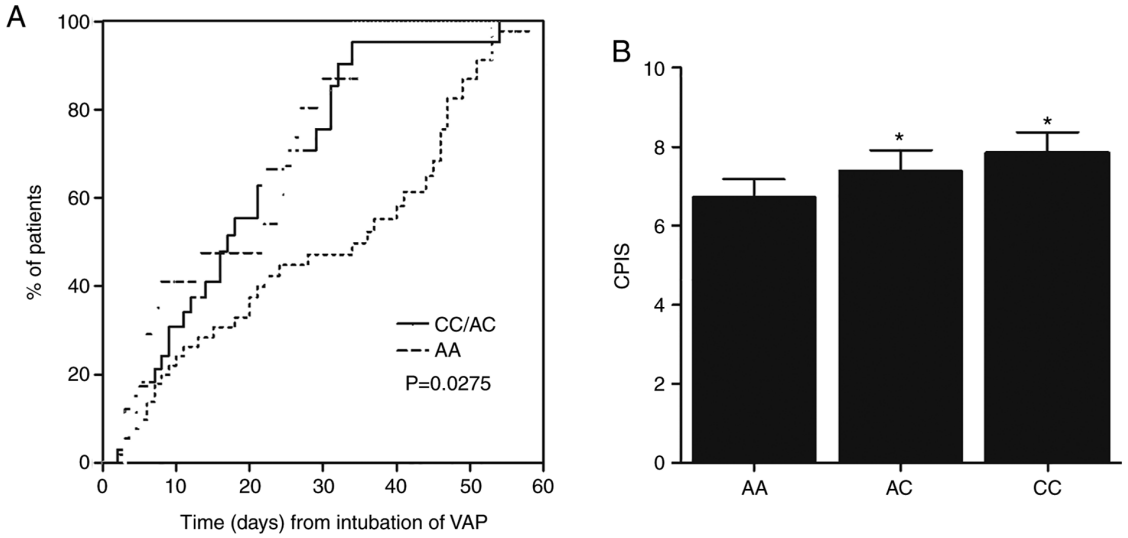

Figure 1. Higher risk of developing VAP in patients with COPD carrying the rs1056629-C allele. (A) Cumulative time to the onset of VAP in patients with COPD carrying the CC, CA and AA genotypes of rs1056629 SNP. (B) CPIS of patients with VAP carrying the CC, CA and AA genotypes of rs1056629 SNP. *P<0.05 vs. AA. VAP, ventilator-associated pneumonia; COPD, chronic obstructive pulmonary disease; CPIS, clinical pulmonary infection score.

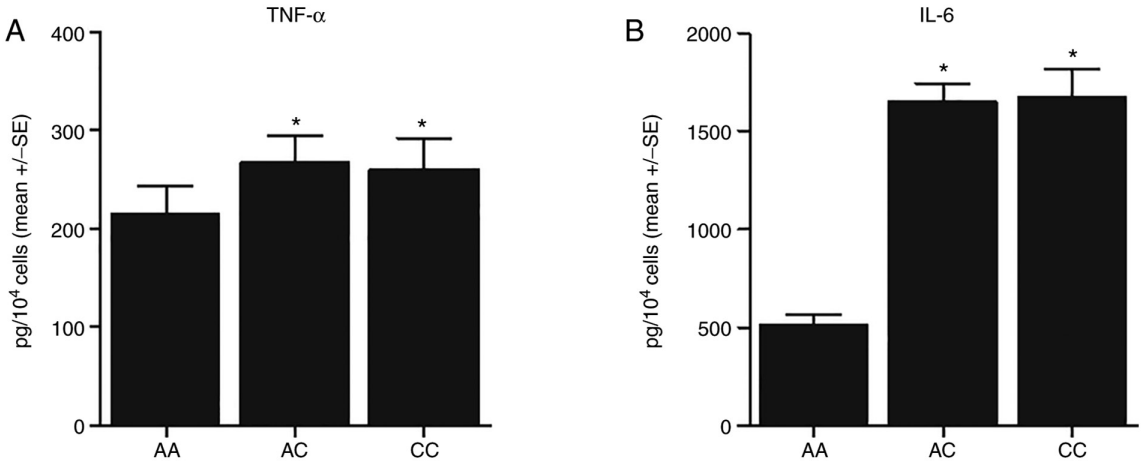

Figure 2. Elevated expression of TNF-α and IL-6 in the monocytes of patients with VAP carrying the rs1056629-C allele. (A) The expression of TNF-α was increased in the monocytes of patients with VAP carrying the CC and CA genotypes of rs1056629 SNP. (B) The expression of IL-6 was elevated in the monocytes of patients with VAP carrying the CC and CA genotypes of rs1056629 SNP. *P<0.05 vs. AA. VAP, ventilator-associated pneumonia.

SNP were constructed, and were then co-transfected with miR-491 into A549 cells (Fig. 4A). The luciferase activity of wild-type rs1056629-A, but not that of mutant rs1056629-C SNP was markedly inhibited by miR-491 (Fig. 4B). Furthermore, the inhibitory effect of miR-491 on MMP-9 expression was also confirmed in H1299 cells (Fig. 4C). Thus, these results indicated that miR-491 suppressed the expression of MMP-9 by binding to its 3'UTR.

Overexpression of miR-491 in A549 and H1299 cells suppresses the expression of MMP-9. To further confirm the inhibitory role of miR-491 in MMP-9 expression, 50 and 100 nM miR-491 mimics were transfected into the A549 and H1299 cells, and this led to the overexpression of miR-491 (Figs. 5A and 6A) in a concentration-dependent manner. MMP-9 mRNA expression was also significantly reduced in the A549 (Fig. 5B) and H1299 cells (Fig. 6B) transfected with miR-491 in a concentration-dependent manner. Similarly, the expression of MMP-9 protein in the A549 (Fig. 5C) and H1299 (Fig. 6C) cells transfected with miR-491 was also decreased in a concentration-dependent manner. Collectively, these results suggested that miR-491 inhibited MMP-9 expression in a concentration-dependent manner.

Discussion

As a type of severe infectious disease, VAP can be easily observed in patients in the ICU who have been treated using mechanical ventilators for at least 48 h (19). As a result, the development of effective measures with the potential to reduce the risk of VAP has become a great challenge (20).

The rs1056629 SNP found in both the seed sequence of miR-491, as well as the 3'UTR domain of MMP-9 is suspected to increase the risk of atherosclerotic cerebral infarction (10). By playing an essential role in the degradation and decomposition of type V and type IV collagens, MMP-9 expression

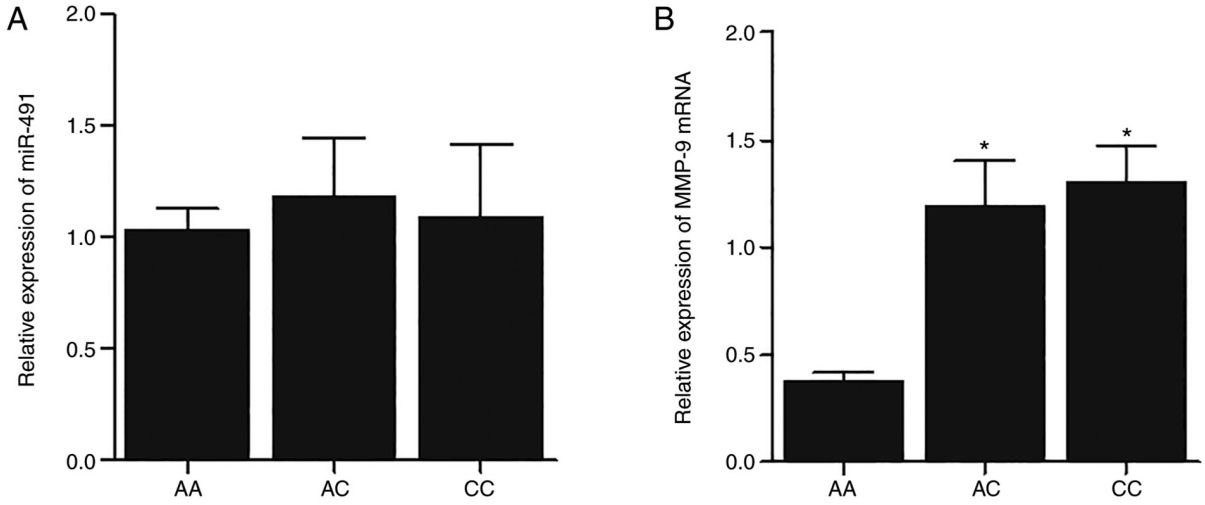

Figure 3. Upregulation of MMP-9 in the monocytes of patients with VAP carrying the rs1056629-C allele. (A) There was no marked difference in the expression of miR-491 among patients carrying different genotypes of rs1056629 SNP. (B) The expression of MMP-9 was increased in the monocytes of patients with VAP carrying the CC and CA genotypes of rs1056629 SNP. *P<0.05 vs. AA. VAP, ventilator-associated pneumonia.

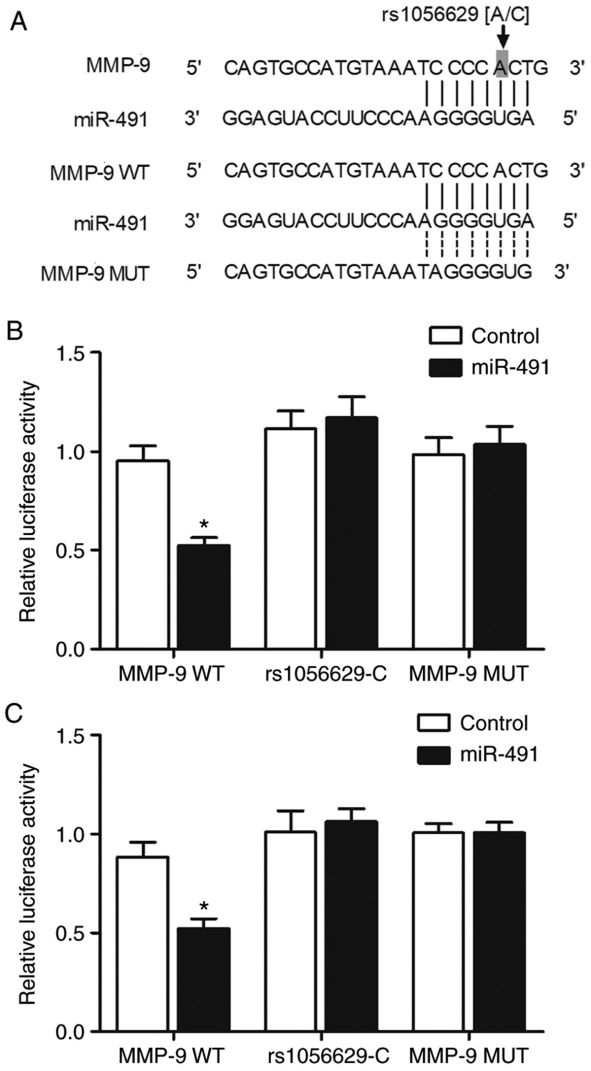

Figure 4. miR-491 inhibits the luciferase activity of the MMP-9 3'UTR containing the rs1056629-A allele. (A) Sequences of luciferase reporter plasmids. (B) The luciferase activity of MMP-9 plasmid containing the rs1056629-A allele was inhibited by miR-491 in A549 cells. (C) The luciferase activity of MMP-9 plasmid containing the rs1056629-A allele was inhibited by miR-491 in H1299 cells. *P<0.05 vs. control.

is increased in patients suffering from acute ischemic stroke, as well as atherosclerosis (21,22). MMP-9 can also play an important role in the onset of vascular remodeling, atherosclerosis, as well as in the formation of arterial plaques (12,23,24). The 3'UTR domain of mRNAs is crucial for maintaining the stability of their host mRNAs while serving as a primary target in the functioning of miRNAs. Nevertheless, a few studies have tried to investigate the regulatory association between the risk of ACI and the presence of various SNPs in MMP-9 3'UTR, although *in vitro* experiments have provided some evidence supporting the aforementioned association (10). In the present study, 96 patients with COPD who developed VAP were enrolled and divided into different groups according to their genotypes of rs1056629 SNP in the 3'UTR of MMP-9. It was found that carriers of rs1056629-C SNP exhibited a significantly accelerated development of VAP. Furthermore, ELISA was performed to evaluate the expression of TNF-α and IL-6 in the monocytes of these patients with VAP, and an elevated expression of TNF-α and IL-6 was observed in patients carrying the rs1056629-C allele. In addition, RT-qPCR was used to assess the differential expression of miR-491 and MMP-9 in the monocytes of patients with VAP carrying different genotypes of rs1056629, and no difference in the expression of miR-491 was observed among the different groups of patients. However, MMP-9 expression was increased in patients carrying the rs1056629-C allele.

Multiple inflammatory diseases, such as ulcerative colitis, have been shown to display an abnormal level of MMP-9 activity and expression (25-29). As a frequently recurring autoimmune disease of the colon, ulcerative colitis is featured not only by a significantly elevated level of MMP-9 proteins, but also a significantly elevated level of proteolytic activity, which may be caused by the excessive expression of certain inflammatory factors, including IL1-α and TNF-α (29,30-33).

Since MMP-9 is apparently associated with an increased risk of developing pneumonia, it has been hypothesized that an elevated level of MMP-9 may be an important contributor for the onset of VAP (14). In the present study, luciferase assays were used to explore the inhibitory effects of miR-491 on MMP-9

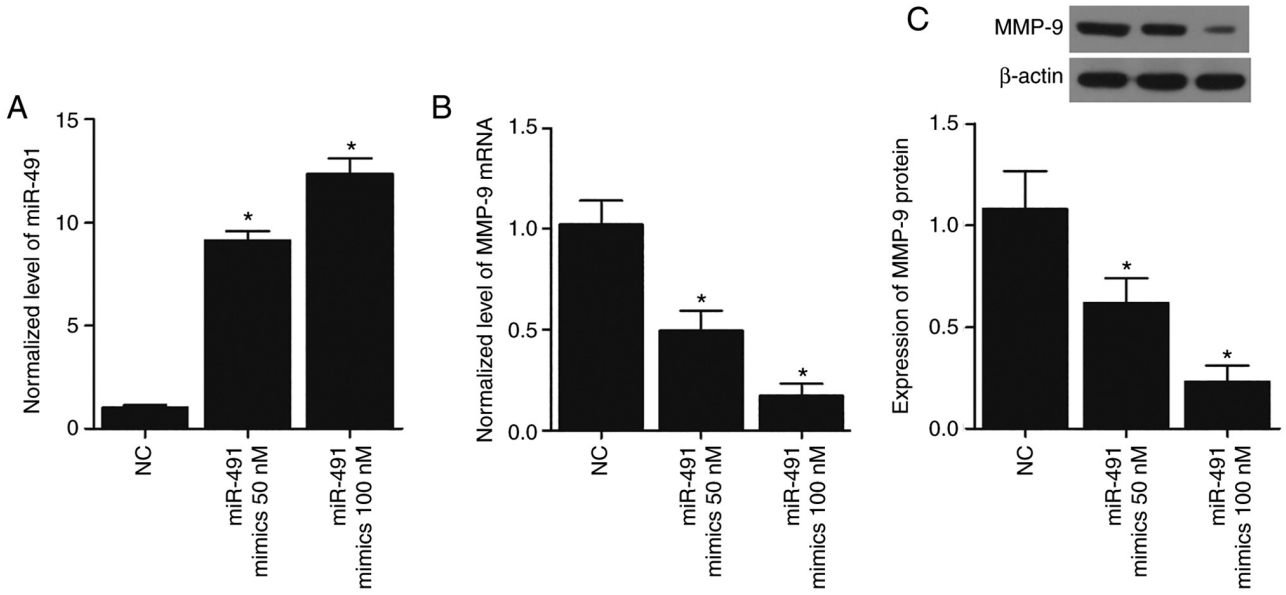

Figure 5. Transfection of miR-491 mimics inhibits the expression of MMP-9 in A549 cells in a concentration-dependent manner. (A) Overexpression of miR-491 in A549 cells. (B) Stronger inhibition of MMP-9 mRNA expression by a higher concentration of miR-491 mimics. (C) Stronger inhibition of the MMP-9 protein expression by a higher concentration of miR-491 mimics. *P<0.05 vs. NC.

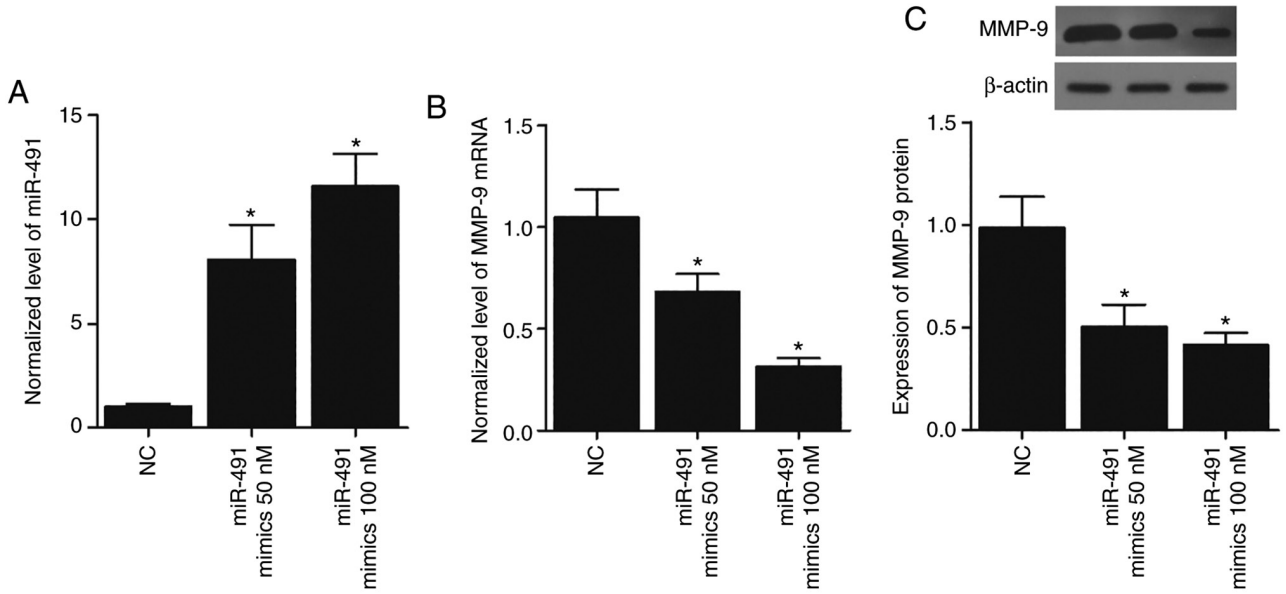

Figure 6. Transfection of miR-491 mimics inhibits the expression of MMP-9 in H1299 cells in a concentration-dependent manner. (A) Overexpression of miR-491 in H1299 cells. (B) Stronger inhibition of MMP-9 mRNA expression by a higher concentration of miR-491 mimics. (C) Stronger inhibition of the MMP-9 protein expression by a higher concentration of miR-491 mimics. *P<0.05 vs. NC.

expression. MMP-9 expression was markedly suppressed by miR-491 overexpression, which bound to the 3'UTR of MMP-9.

The activity of MMP-9 is apparently increased in patients with pneumonia (34). In addition, the level of MMP-9 has been shown to be markedly increased in patients with bronchoalveolar lavage fluid (BALF) along with an obviously increased number of apoptotic neutrophils (35,36). Furthermore, the plasma level of MMP-9 proteins in patients with VAP is much higher than that in patients free of VAP, while the application of appropriate treatments significantly decreases the level of MMP-9 (11). In the present study, A549 and H1299 cells were transfected with miR-491 mimics, and the mRNA and protein expression of MMP-9 was then analyzed by RT-qPCR and western blot analysis, respectively. It was found that MMP-9 expression in the A549 and H1299 cells was markedly decreased by miR-491 overexpression.

MMP-9 can be a main contributor to the induction of inflammatory responses by acting to cleave pro-inflammatory factors, such as IL-1β (37-40). In addition, MMP-9 can also promote the undocking of activated TNF from the cell plasma membrane to facilitate the induction of inflammation (41-43). During the pathogenesis and development of myocardial infarction, MMP-9 is released by both macrophages and neutrophils to participate in the degradation of the extracellular matrix, as well as the

regulation of TGF-β functions, which have been shown to play an essential role in the formation of collagenous scars, as well as in the pathogenesis of myocardial fibrosis (44,45).

In conclusion, the present study demonstrated that the rs1056629 SNP located in MMP-9 3'UTR affected the risk of developing VAP. The possible mechanisms underlying such observations are that the C-to-A substitution of rs1056629 SNP affects the binding of miR-491 to MMP-9 3'UTR, leading to MMP-9 overexpression and a higher risk of developing VAP. Thus, the rs1056629 SNP of MMP-9 may be used as an important biomarker to determine the susceptibility to VAP.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WM and XL designed the study. XC, WS and LZ performed the literature search for the study. All authors (WM, XC, WS, LZ, BF, SZ, HL, HW, WW and XL) performed the experiments and collected the data. XC, BF, SZ and HL analyzed the data and prepared the figures. WW, WM, XC and XL composed the manuscript. HW, WW and WS corrected the language. WM and XL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the study, as well as the template of informed consent form (ICF) was reviewed and approved by the Clinical Ethics Committee of our Qinghai Red Cross Hospital for the retrospective use of these blood samples, and written informed consent was obtained from all subjects or their family members prior to the initialization of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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