

Original Article

Expression of MMP-9/TIMP-2 in nasal polyps and its functional implications

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Abstract: Nasal polyps (NP) involve tissue repair and structural remodel, both of which require the extracellular matrix. Matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) are known regulators for tissue reconstruction. This study therefore aimed to analyze the expressional profile of MMP-9 and TIMP-2 in NP patients, with further investigation of their roles in pathogenesis. A total of 60 NP tissue samples (including 15 type I, 21 type II and 24 type III) were collected from surgeries in our hospital, in addition to 6 normal ethmoid sinus mucosa samples. The mRNA and protein expression levels of MMP-9/TIMP-2 were quantified by real-time PCR and Western blotting, respectively. Serum levels were also checked by enzyme-linked immunosorbent assay (ELISA). Both mRNA and protein levels of MMP-9 in NP tissues or serum were significantly elevated compared to those in control ones ($P<0.05$) while the TIMP-2 expression was suppressed ($P<0.05$). In patients with more advanced stage, MMP-9 expression was further elevated, with lowered TIMP-2 levels ($P<0.05$ in both cases). Pathogenesis and progression of NP is closely related with elevated MMP-9 and suppressed TIMP-2 expression, suggesting the role of those factors as indexes for evaluating NP stage. Our results also provide evidences for further studies of pathogenesis and drug targets of NP.

Keywords: Nasal polyps, chronic sinusitis, MMP-9, TIMP-2

Introduction

As a common chronic disease, nasal polyps (NP) are manifested as persistent course and frequent recurrence, thereby severely affecting the life quality of patients [1]. The pathogenesis mechanism has not been fully illustrated so far, but most scholars agree that a cascade involving various factors, genes and signals are involved. In brief, pathogens including fungi, virus and bacteria stimulate the nasal mucous inflammation, which may increase the vascular permeability, leading to tissue edema and degradation. Such damages of epithelial tissues can cause the reformation of epithelial layers involving the *de novo* formation of glands and vessels, finally leading to NP [2-4]. Therefore pathogenesis of NP is dependent on structural remodeling [5], which involves the participation of extracellular matrix (ECM) including collagen and fibrin and inflammatory factors. The crucial role of ECM in tissue remodeling of both normal tissues and pathological polyps has been reported [6, 7].

Matrix metalloproteinase (MMP) is a family of calcium-dependent endogenous proteinase and can degrade protein components of ECM and basal membrane, thereby playing a key function in the homeostasis of ECM under normal physiological conditions [8, 9]. MMP can be specifically inhibited by tissue inhibitor of metalloproteinase (TIMP) [10]. Thus both MMP and TIMP maintain the integrity of ECM and cellular basal membrane via the dynamic balance of those two factors. The pathological tissue remodeling may occur as a result of imbalance between MMP and TIMP, both of which break the integrity of ECM and basal membrane, thereby impeding the normal tissue repair in various diseases including auto-immune dysfunction, tumor metastasis and inflammation [11, 12]. However, no study has been performed regarding the expression of MMP-9 and TIMP-2 in NP and their implications. This study therefore aimed to investigate the role of those two factors in disease pathogenesis via the expressional quantification.

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Table 1. Real-time PCR primer sequences

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|--------|---------------------------|---------------------------|
| GADPH | AGTGC CAGCC TCGTC TCATAG | CGTTG AACTT GCCGT GGGTA G |
| MMP-9 | GATCT ACGCA GCGAA GAACT T | CTCTG GGAC ATCTC CGTCA |
| TIMP-2 | CTACG GAAGA TCTCA ATAGC G | GGGAC TCTCA ATCCT CGTC |

Table 2. General information of NP patients

| Subtype | N | Sex (M/F) | Age (yrs) |
|----------|----|-----------|-------------|
| Type I | 15 | 8/7 | 37.24±17.65 |
| Type II | 21 | 10/11 | 39.1±20.36 |
| Type III | 24 | 11/13 | 38.1±23.21 |

Methods and materials

Research subjects and sampling

A total of 60 NP patients (29 males, 31 females; aging between 26~60 years old, average=38.2±22.7 years old) admitted in our hospital between Jan 2013 and Dec 2014 were recruited in this study. Clinical grading was performed based on nasal endoscopy and nasal sinus CT scan results, along with patient's medical history [13]. There were 15 cases of type I, 21 type II and 24 type III cases. Tissue samples were collected from inflammatory mucous of type I lesion and polyps of type II/III lesion. Meantime, 6 normal sinus mucosa tissue samples were collected from nasal septal reconstruction surgery as control group. Samples were collected, divided into small pieces (1 mm³) and stored in liquid nitrogen. All patients had not received any glucocorticoid medication within 4 weeks before the surgery. General information including sex and age had no significant difference across all groups ($P>0.05$). This study has been pre-approved by the ethical committee of our hospital and written consents have been obtained from all participants.

Western blotting for MMP-9 and TIMP-2 protein quantification

Tissues were lysed on ice for 30 min using lysis buffer (Beyotime Biotech, China). Cells were further ruptured in ultrasound (5 sec, 4 times) and were centrifuged at 10 000 g for 15 min. Supernatants were transferred to new tubes and quantified. 10% SDS-PAGE was used to separate proteins, which were transferred onto PVDF membrane (Pall Life, US) by semi-dry

method. Non-specific binding sites were blocked by 5% defatted milk powders for 2 hour at room temperature. The membrane was then incubated with rabbit primary antibody against MMP-9 (1:500, DPC Biermann, Germany), TIMP-2 (1:1000, DPC Biermann, Germany) or β -actin (1:1000, Santa Cruz, US) overnight. After washing with PBST, mouse anti-rabbit IgG conjugated with horseradish peroxidase (1:2000, Cell signaling, US) was added for 30-min incubation. Chemical chromogenic substrates (Beyotime Biotech, China) were used to develop the membrane for 1 min, followed by X-ray visualization. The images were captured by Quantity One software (BioRad, US). Relative optical density (OD) values were measured by Image J software. β -actin was used as an internal reference. All experiments were repeated for four times (N=4) for statistical analysis.

Real-time PCR for mRNA quantification

NP tissues were intensively grinded and were eluted repeatedly using 150 μ l buffer until obtained a clear suspension. Tissue elute was further centrifuged at 10,000 g for 2 min and added with Trizol (Invitrogen, US) to extract total RNA according to the manual instruction. cDNA was synthesized using specific primers (**Table 1**). Target gene was quantified using real-time PCR in buffers (Invitrogen, US) using the following condition: 52°C for 1 min; 90°C denature for 30 sec, 58°C annealing for 50 sec, plus 72°C elongation for 35 sec. The cycle repeated for 35 times. Fluorescent quantitative PCR reactor collected the data. Standard curves were plotted using internal reference gene GAPDH. Relative mRNA levels were determined using 2^{- Δ Ct} method.

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood samples were collected from elbow veins before the surgery. After centrifugation at 3,000 g for 15 min, serum was transferred to new tubes and stored at -80°C. ELISA was used to quantify serum MMP-9 and TIMP-2 levels using test kits (RD, US). In brief, 50 μ l serial diluted standard samples were tested to plot the standard curve. Samples were then added into 96-well plate. After gentle washing, 50 μ l enzyme-linked reagents were added into

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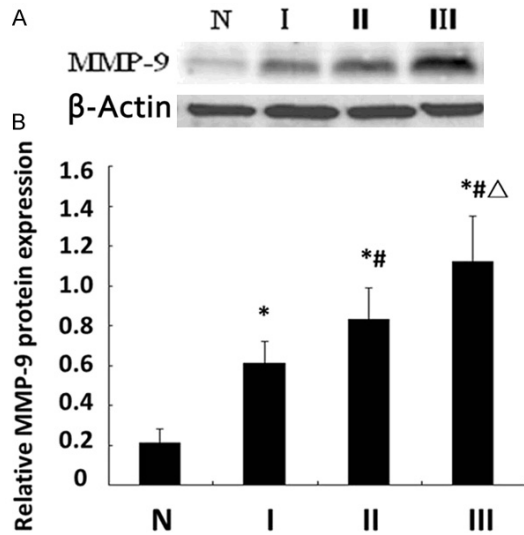


Figure 1. Expression of MMP-9 in NP tissues. A. Showed representative bands of Western blotting; B. Showed quantified expression levels of MMP-9 proteins (using actin as the normalized level). N, control group; I, II and III represented sub-types of NP. *, $P < 0.05$ compared to control ones; #, $P < 0.05$ compared to type I; Δ, $P < 0.05$ compared to type II.

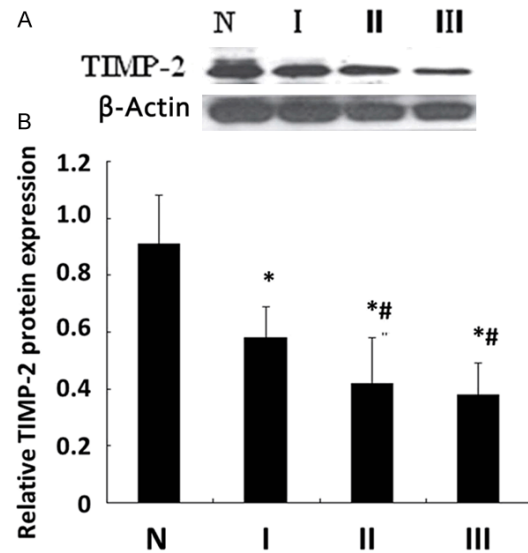


Figure 2. Expression of TIMP-2 in NP tissues. A. Showed representative bands of Western blotting; B. Showed quantified expression levels of TIMP-2 proteins (using actin as the normalized level). N, control group; I, II and III represented sub-types of NP. *, $P < 0.05$ compared to control ones; #, $P < 0.05$ compared to type I.

each well, followed by incubation at 37°C for 30 min. After washing, chromogenic substrate A and B were added into each well. The reaction was stopped after 10-min incubation by stopping buffer. OD values at 450 nm were measured in each well. Concentration of samples was deduced based on the standard curve.

Statistical analysis

All collected data were processed using SPSS 13.0 software package. Numeration data were tested by chi-square method while measurement data were expressed as mean \pm standard deviation (SD). Analysis of variance (ANOVA) was employed to compare data across multiple groups. A statistical significance was defined when $P < 0.05$.

Results

General information of NP patients

Similar onset ages existed across different types of NP patients. Overall speaking, the average age is 38.2 ± 22.7 years old, with similar incidence between males and females (Table 2). All those parameters showed no significant difference ($P < 0.05$).

Expression of MMP-9 proteins

Western blotting showed lower MMP-9 proteins in normal mucous tissues. In NP samples, there were significantly more MMP-9 proteins ($P < 0.05$). Further comparisons across different sub-types of NP showed that type III NP tissues had significantly more MMP-9 compared to those in type II, which are higher than type I (Figure 1, $P < 0.05$ in both cases).

Expressional profiles of TIMP-2 proteins in NP

A similar study was also performed on TIMP-2 protein levels. Results (Figure 2) showed an opposite pattern of this protein when compared to MMP-9: the highest level of TIMP-2 occurred in normal tissues, and was gradually decreased in type I, II and III NP samples. Statistical analysis showed that type I NP tissues had significantly lowered TIMP-2 proteins compared to control ones ($P < 0.05$). Type II/III NP had significantly suppressed TIMP-2 when compared to type I patients ($P < 0.05$). No significant difference, however, was identified between type II and III tissues ($P > 0.05$). Such results of TIMP-2 along with MMP-9 proteins as abovementioned illustrated the occurrence of potentiated

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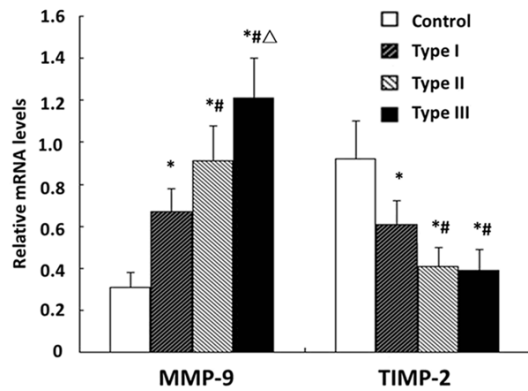


Figure 3. mRNA levels of MMP-9 and TIMP-2 in NP tissues. *, $P < 0.05$ compared to control ones; #, $P < 0.05$ compared to type I. Δ, $P < 0.05$ compared to type II.

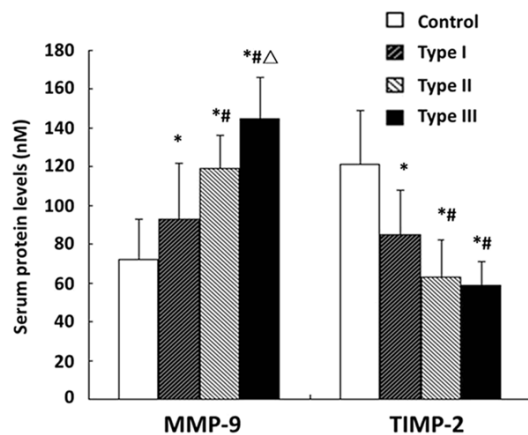


Figure 4. Serum levels of MMP-9 and TIMP-2 in NP tissues. *, $P < 0.05$ compared to control ones; #, $P < 0.05$ compared to type I. Δ, $P < 0.05$ compared to type II.

MMP-9 and suppressed TIMP-2 expression during the occurrence of NP.

mRNA levels of MMP-9 and TIMP-2 in different subtypes of NP

We further extracted mRNA from NP tissue samples and quantified their expressions of MMP-9 and TIMP-2 by semi-quantitative real-time PCR. Results (Figure 3) showed consistent patterns with those in Western blotting: MMP-9 mRNA levels were gradually potentiated in type I, II and III NP patients, when compared to normal ones ($P < 0.05$ in all paired comparisons). TIMP-2 mRNA level, however, was significantly decreased in NP tissues ($P < 0.05$).

Serum MMP-9 and TIMP-2 levels in different subtypes of NP patients

We further used ELISA to quantify the serum protein levels of MMP-9 and TIMP-2 in different NP patients. As shown in Figure 4, MMP-9 levels in NP patients were significantly elevated compared to control ones ($P < 0.05$), with significant difference across different subtypes. Meantime, TIMP-2 levels were significantly suppressed in all NP patient's serum ($P < 0.05$). All those patterns were consistent with those in Western blotting and real-time PCR, thereby suggesting that the dynamic balance between MMP-9 and TIMP-2 was impaired in NP, suggesting the involvement of tissue repair and reconstruction. Our results also point the potency of those two indexes as reference for clinical diagnosis.

Discussion

NP is manifested with nasal congestion, headache and dysosmia. It can aggravate the respiratory infection symptoms, compromising patient's life quality or even inducing intracranial complications [13]. Classical views believe that nasitis, nasosinusitis and NP are three independent diseases. Recent pathological studies, however, suggested the correlation among those diseases as they share the common disposing factor of inflammation. Moreover, there is a gradual progression from nasitis to nasosinusitis and NP. In brief, NP originated from nasitis, which may develop edema of mucous, vessel proliferation, increased permeability and formation of glands, contributing to the inflammatory lesion, i.e. nasal polyps [14, 15]. However, no clear explanation of NP's pathogenesis has been reported so far. Most scholars believe that NP is caused by a cascade reaction involving multiple factors including bacteria, virus, allergen and air pollution, all of which activate epithelial cells and stimulate the release of inflammatory cytokines, thereby facilitating infiltration in inflammatory cells [16].

Recent studies showed that the tissue remodel and degradation of basal membrane is one key step in the formation of NP [6, 7]. MMP-9, as one important member of MMP family for the degradation of ECM, exists in various body tissues. Under normal physiological conditions, it can regulate cell-to-cell adhesion via modulating ECM breakdown and tissue remodel by its

actions on substrate type IV collagen [17, 18]. MMP-9 can modulate tissue reconstruction, embryonic development and injury repair by its regulation on extracellular components, with the help of zinc ions [19]. The over-activation of MMP-9, however, may cause the intensive breakdown of ECM, thereby facilitating tumor infiltration/metastasis, and auto-immune disease including osteoarthritis and rheumatoid arthritis. TIMP-2, an endogenous specific MMP inhibitor, can prevent MMP-9 from over-activation; thereby maintain homeostasis of ECM [20].

In this study, we found less MMP-9 but more TIMP-2 proteins in normal tissues. In NP samples, MMP-9 protein level was significantly elevated while TIMP-2 expression was suppressed. Further analysis revealed that with more advanced NP type, MMP-9 expression (both mRNA and protein) was gradually increased while TIMP-2 was decreased. All those results suggest that the facilitation of MMP-9 and inhibition of TIMP-2 can potentiate the disease course. This can be explained as abnormally higher MMP-9 and lower inhibitor TIMP-2 can elevate degradation of ECM, breaking the basal membrane, thereby loosening the connective tissue of nasal lamina propria. All these events can facilitate the formation of edema, break down vascular basal membrane and increase the vessel permeability, thus further aggravating the edema of nasal mucosa and formation of polyps [21, 22]. Further assays in serum also identified similar patterns of those two proteins, confirming the imbalance of MMP-9 and TIMP-2 in NP patients and suggesting the potency of those factors as reference indexes for disease evaluation.

In summary, the occurrence and progression of NP are closely related with elevated MMP-9 and suppressed TIMP-2 proteins. Those two factors may work to evaluate the clinical subtype/stage of NP. Our results also provide evidences for further studies regarding NP pathogenesis and development of drug targets.

Disclosure of conflict of interest

None.

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