



ORIGINAL ARTICLE

Expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 in recurrent chronic rhinosinusitis with nasal polyposis

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Abstract Matrix metalloproteinase (MMP) is involved in the upper airway remodeling process. We hypothesized that MMP had an additive effect on the formation of recurrent nasal polyp. We also investigated the association between the functional promoter polymorphism of MMPs and the intensity of labeling index. Expressions of MMP-2 and MMP-9 were assessed via immunohistochemical staining and compared between different groups, including recurrent nasal polyps, nonrecurrent nasal polyps, and control nasal mucosa. Two promoter functional single-nucleotide polymorphisms (rs3918242 for MMP-9 and rs243865 for MMP-2) were selected to correlate with staining intensity. Expression of MMP-9 was significantly enhanced in gland for recurrent nasal polyp ($p = 0.016$) and nonrecurrent nasal polyp ($p = 0.005$) compared to the control. MMP-2 positivity was significantly increased in surface epithelium for recurrent nasal polyp ($p = 0.004$) compared to the control ($p = 0.061$). However, there was no significant difference in MMP-9 and MMP-2 expressions between recurrent and nonrecurrent nasal polyps. Genetic polymorphism of MMP-2 and MMP-9 functional promoters was not associated with the

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intensity of labeling index. These results suggested that up-regulation of MMP-9 in gland and MMP-2 in surface epithelium was characteristic of both recurrent and nonrecurrent nasal polyps. Pathogenesis of recurrent nasal polyps may involve a mechanism other than MMP. Copyright © 2012, Kaohsiung Medical University. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Nasal polyposis, a subgroup of chronic rhinosinusitis, remains one of the most difficult challenges in clinical rhinology. Its etiology and pathophysiology are still controversial, medical treatment is unsatisfactory, and recurrences necessitate repeated surgical interventions. Histological appearance of nasal polyposis is characterized by inflammatory cell infiltration, modifications of epithelial cell differentiation, and tissue remodeling including basement membrane thickening, gland modifications, extracellular matrix (ECM) accumulation, and edema. The fact that nasal polyposis and asthma share characteristic inflammatory features and histopathologic findings of airway remodeling led researchers to investigate the possibility that the airway remodeling occurring with nasal polyps was similar to that with asthma.

Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases that can collectively degrade almost all ECM components, is important in the process of airway remodeling [1,2]. Among the MMP family, MMP-2 (72-kD type IV collagenase, gelatinase A) and MMP-9 (92-kD type IV collagenase, gelatinase B) more specifically hydrolyze denatured collagens (gelatin); native types IV, V, and XI collagens; and elastin [3–5]. They were characterized as latent zymogens that could be activated *in vitro* by several proteases, via cleavage of propeptides [1,2]. Although the substrate specificities of gelatinases appeared similar, these two enzymes were known to be synthesized by various cells. MMP-2 was constitutively produced by numerous cell types, whereas MMP-9 was produced by inflammatory cells such as macrophages [6], neutrophils [7], and airway epithelial cells [8,9].

Roles of MMPs in lung disease are established. In transgenic animal studies, an elevated level of *MMP-9* and *MMP-2* was associated with defects in bronchial architecture [10]. MMPs produced by inflammatory cells appeared to be responsible for microvascular permeability leading to edema and cell transmigration, and ECM remodeling in asthmatic airways [11–13]. MMPs also played a role in bronchial subepithelial fibrosis in asthmatic airways [13]. Previous studies suggested that polyposis formation involved ECM protrusion through an initial localized epithelial defect [14]. Many studies had also mentioned the occurrence of tissue remodeling and the involvement of MMPs in the formation of nasal polyp, but the results were inconsistent and none of them discussed the expression of MMPs in the recurrent nasal polyp. These findings led us to investigate the relative expression of MMPs in the nonrecurrent and recurrent nasal polyps as compared to control individuals.

Materials and methods

Participants

We recruited 30 fresh patients of bilateral chronic rhinosinusitis with nasal polyposis (CRSwNP) who were admitted to the Kaohsiung Medical University Hospital for endoscopic sinus surgery. The diagnosis of CRSwNP was made on the basis of the definition in European position paper on rhinosinusitis and nasal polyps 2007 (EPOS 2007) [15]. Patients with malignancies or asthma were excluded from the study. After surgery, we routinely used topical steroid spray for at least 6 months postoperatively. Nasal saline irrigation was also advised and carried out in the patients. All the patients were followed weekly for the first month and then monthly for at least 6 months postoperatively. None had recurrent nasal polyp 6 months after operation. Recurrence of nasal polyp was defined as newly developed pedunculated nasal polyps (instead of cobblestone or polypoid mucosa) fully occupying the middle meatus in a patient 6 months after surgery, as seen by either anterior rhinoscope or nasal endoscope. Recurrence was identified in 32 patients for whom revision surgery was performed at our institution. Thirty-one patients with chronic rhinitis with septal deviation admitted for septoplasty were recruited as controls. Inferior turbinate mucosa from control individuals was used for immunohistochemical (IHC) staining. None of the controls reported major disabling diseases upon enrollment. Information on demographic characteristics was collected. The study was approved by the Institutional Review Board (IRB) of our hospital (KMUH-IRB-940216), and written informed consent was obtained from each participant.

SNP selection and genotyping

Genomic DNA was extracted from peripheral blood by a standard method. Two promoter functional polymorphisms were selected from the HapMap project (International HapMap Consortium), both with the minor allele frequency $\geq 10\%$ in the Han Chinese population. The two promoter functional single-nucleotide polymorphisms (SNPs) are MMP-9 promoter functional SNP (rs3918242, i.e., -1562 C/T) and MMP-2 promoter functional SNP (rs243865, i.e., -1306 A/G). Genotyping for the two SNPs was carried out by using the TaqMan 5' nuclease assay (Applied Biosystems, Foster City, CA, USA). The details were given in our previous studies [16,17].

IHC staining for MMP-2 and MMP-9

IHC analysis was performed on 4- μ m-thick paraffin sections. Paraffin sections of all samples were deparaffinized,

rehydrated, and autoclave-treated at 121°C for 10 minutes in DAKO Target Retrieval Solution, pH 9.0 (DAKO, Glostrup, Denmark), to induce antigen retrieval. Endogenous peroxidase in the section was blocked by incubation in 3% hydrogen peroxide for 5 minutes. After blocking with 0.5% goat serum for 60 minutes, the sections were incubated with primary antibodies MMP-2 and MMP-9 (NeoMarkers, Union City, CA, USA) at room temperature for 1 hour. Then, the DAKO REAL EnVision Detection kit (DAKO) was applied for 30 minutes. Finally, sections were incubated in 3',3'-diaminobenzidine for 5 minutes, followed by Mayer's hematoxylin counterstaining and mounting. Negative controls were obtained by replacing the primary antibody with nonimmune serum. Samples of breast carcinoma known to produce MMP-2 and MMP-9 were used as positive controls. MMP-2 and MMP-9 expression in nasal polyp and inferior turbinate mucosa were evaluated and quantified in surface epithelium and gland. The methods to quantify MMP labeling index were identical to those described by Lechapt-Zalcman et al. [18]. Epithelial labeling indices were estimated as the percentage of positive surface epithelial fields over the total number of surface epithelial fields present on each slide (final magnification 200×). For inferior turbinate mucosa, images of about 20 consecutive high-power fields were obtained and the positively stained epithelial fields were counted. For nasal polyps, images of about 40 consecutive high-power fields were obtained and the positively stained epithelial fields were counted. Gland labeling indices were expressed as the percentage of glands with positive cells over the total number of glands counted in 10 randomly selected high-power fields (final magnification 200×). For inferior turbinate mucosa, images of about 80 glands were obtained and the positively stained glands were counted. For nasal polyps, images of about 40 glands were obtained and the positively stained glands were counted.

Statistical analysis

ANOVA with *post hoc* test was used for statistical analysis (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA). Chi-square test was used to calculate the relationship between results of IHC staining and genotyping. A *p* value of less than 0.05 was considered to be statistically significant.

Results

Results of IHC staining

In all samples, cytoplasmic immunostaining for MMP-2 and MMP-9 was observed in the surface epithelium, glands, and connective tissue. In the surface epithelium, MMP-2 positivity was present in basal cells and in ciliated cells, where it was concentrated in the apical part of their cytoplasm, while MMP-9 immunolabeling was detected mainly in the basal cells. Intense staining of MMP-9 was also detectable in polymorphonuclear cells. The differences in the intensity of IHC staining for each group are detailed in Figs. 1 and 2. Comparison of IHC labeling index in the recurrent nasal polyp and control nasal mucosa showed a significant increase in the surface epithelium for MMP-2 ($p = 0.004$), and a significant increase in the gland for MMP-9 ($p = 0.016$). Comparison of IHC labeling index in the

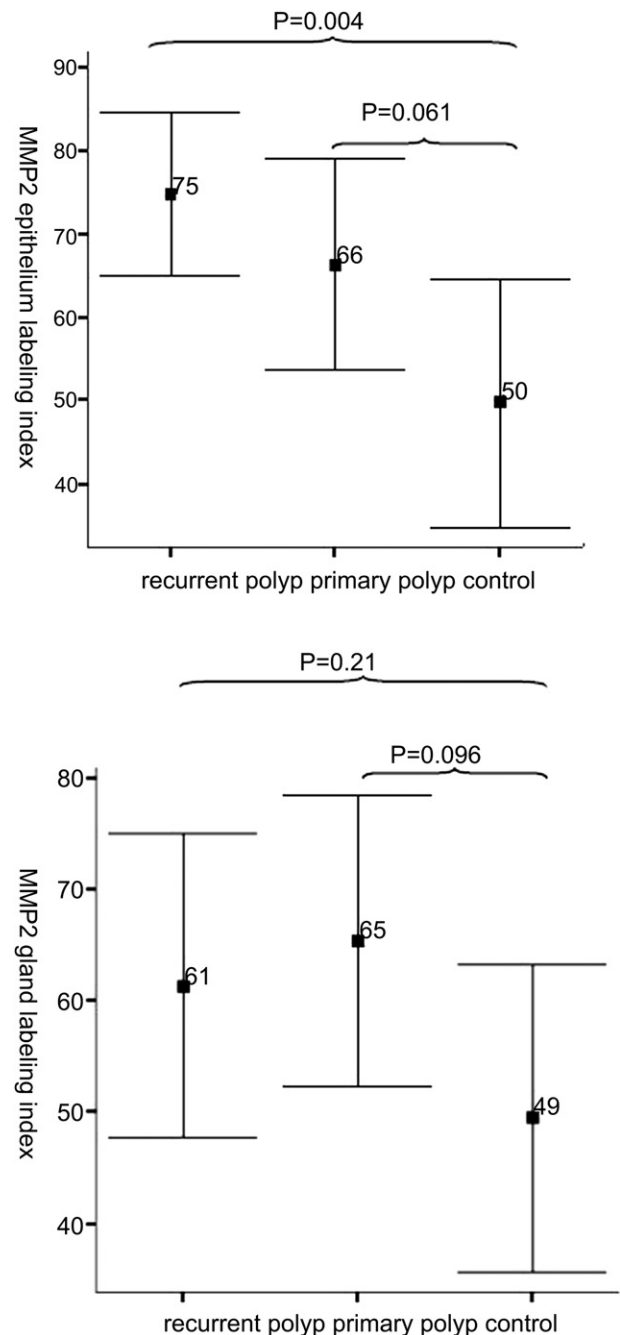


Figure 1. MMP-2 labeling index in epithelium and gland in each group.

nonrecurrent nasal polyp and control nasal mucosa also showed a significant increase in the gland for MMP-9 ($p = 0.005$) and a *p* value toward significance in the surface epithelium for MMP-2 ($p = 0.061$). However, there was no significant difference in MMP-2 and MMP-9 labeling indices between nonrecurrent and recurrent nasal polyps in either surface epithelium or gland.

Result of genotyping

Results of genotype for these two promoter functional SNPs and their relation to the intensity of MMP-2 and MMP-9 IHC

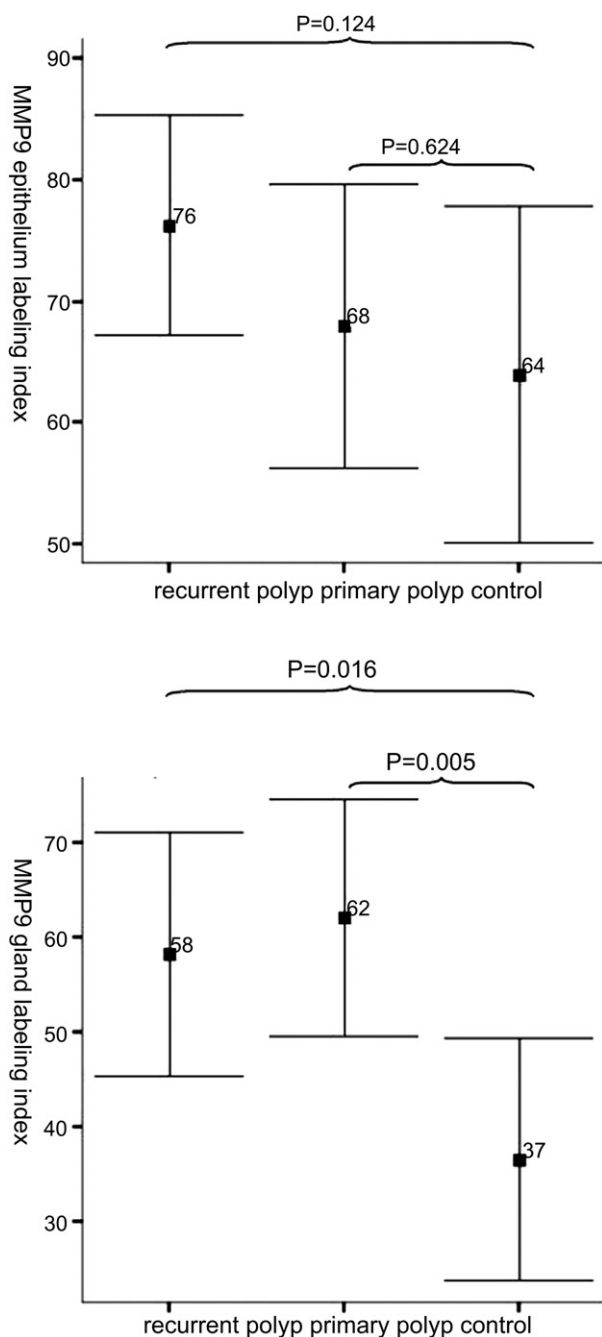


Figure 2. MMP-9 labeling index in epithelium and gland in each group.

staining are shown in Table 1. The difference in the intensity of IHC staining for each allele did not reach statistical significance.

Discussion

To the best of our knowledge, this is the first study to investigate the expression of MMP-2 and MMP-9 in recurrent CRSwNP. This study compared the relative protein expression of MMP-2 and MMP-9 in nonrecurrent nasal polyp, recurrent nasal polyp, and control mucosa from inferior

Table 1 Relationship between MMP-2 and MMP-9 genotype and intensity of labeling index in epithelium and gland in each group.

| Subgroup | MMP-9 rs3918242 genotype | | | MMP-2 rs243865 genotype | | |
|----------------------|--------------------------|-------|------|-------------------------|----|----|
| | CC | CT | TT | GG | GA | AA |
| LI in epithelium (%) | | | | | | |
| r-Polyp | 78.75 | 72.14 | 92.5 | 78 | 74 | NA |
| m-Polyp | 67.75 | 66.25 | 85 | 66 | 30 | 90 |
| Control | 66 | 62 | 90 | 55 | 29 | NA |
| LI in gland (%) | | | | | | |
| r-Polyp | 61 | 44 | 90 | 65 | 53 | NA |
| m-Polyp | 61.5 | 65.56 | 75 | 65 | 30 | 93 |
| Control | 40 | 23.75 | 10 | 55 | 37 | NA |

LI was expressed as the percentage of epithelium (or glands) with positive cells over the total number of cells in epithelium (or glands). All $p > 0.05$ and do not reach statistical significance, which means that the genotype does not correlate with the LI of MMPs in either epithelium or glands. LI = labeling index; m-polyp = nonrecurrent polyp; NA = nonapplicable; r-polyp = recurrent polyp.

turbinate. Distribution of MMP-2 and MMP-9 in nasal polyps is demonstrated by immunohistochemistry using specific antibodies. We showed that both control mucosa and nasal polyp intensely expressed MMP-2 and MMP-9. MMP-9 expression was significantly enhanced in the gland in recurrent nasal polyps and MMP-2 expression was significantly enhanced in the surface epithelium in recurrent nasal polyps, both compared with control mucosa. As expected, MMP-9 was expressed mainly in inflammatory cells, but this was also the case in epithelial cells. We also observed intense MMP-2 and MMP-9 expression in the glands and vessels, which was characteristic of nasal polyps.

The relevance of MMP-2 to nasal polyp is controversial. The presence of MMP-2 in nasal polyp was demonstrated in several studies [18–21], but only Bhandari et al. [20] detected a significantly higher MMP-2 mRNA expression in nasal polyp compared to the control tissue (inferior turbinate). MMP-2 is known to be produced by activated fibroblasts, and its role in tissue repair and remodeling within the respiratory epithelium has been proved *in vitro* [8]. Surface epithelium injured by inflammation might be a potential source of MMP-2. This hypothesis is supported by our IHC results, which showed that injured respiratory epithelium in recurrent nasal polyp expressed significantly higher MMP-2 as compared to the control mucosa. There was also a p value near significance ($p = 0.061$) when comparing MMP-2 labeling index in surface epithelium in nonrecurrent nasal polyps to that in the control.

The presence of MMP-9 in nasal polyp is also a controversial issue. Clinically, patients with poor-healing reaction after sinus surgery had more severe edematous and fibrotic changes [22], and higher amounts of MMP-9 in both nasal secretions and connective tissue [23] when compared with good healers. Bhandari et al. [20] stated that they could not find any MMP-9-positive cells in either nasal polyp or inferior turbinate mucosa. Some previous studies showed that

MMP-9 was expressed in both surface epithelium and gland, and its expression was significantly higher in nasal polyp [24–26]. In our study, MMP-9 was significantly enhanced in the gland, but not in the surface epithelium, of recurrent nasal polyps as compared to the control. This supported a relationship between MMP-9 and ECM remodeling in nasal polyp. The formation of nasal polyp glands has been well described histologically, but the cellular mechanisms remain poorly understood. The “epithelial rupture theory” postulated that polyp formation results from the protrusion of connective tissue through an initial epithelial defect, which is subsequently re-epithelialized [27]. Gland buds could be formed within this area of re-epithelialization by epithelial basal cell proliferation and migration into the underlying connective tissue. As has already been shown for tracheal gland morphogenesis and ureter bud branching, some morphological findings suggest the involvement of MMPs in nasal polyp gland formation [28,29]. Most nasal polyp glands were either cystically dilated or elongated tubular glands, suggesting a remodeling and developing process. Enhanced MMP-9 expression in the gland supports the hypothesis that MMPs are involved in these morphological changes.

In this study, we demonstrated that MMPs were highly expressed in chronic inflammation of the sinuses as compared to control nasal mucosa. We suggest that this release pattern of enzymes, released by inflammatory cells in the pseudocyst formations as well as vascular, epithelial, and glandular elements, is characteristic of nasal polyp. However, we found no difference between nonrecurrent and recurrent nasal polyps. In other words, we were unable to demonstrate that the MMP-2 and MMP-9 had more up-regulation in the process of recurrent nasal polyp.

Regulation of MMPs is complex. It is considered that regulation of MMP activity occurs at three levels: gene transcription, activation of the secreted proenzyme, and inhibition by specific and nonspecific inhibitors [30]. Furthermore, MMP-9 protein [24,26,31,32], MMP-9 mRNA [24,26], MMP-2 protein [19,20], and MMP-2 mRNA [20] levels were reported to be different in patients with the CRSwNP. Although Zhang et al. [33] reported that the T allele of promoter SNP rs3918242 at MMP-9 had a higher promoter activity, which would lead to a higher production of MMP-9, neither a recent study [34] nor our results found any differential expression between the T and C alleles. Our results also showed that there was no significant difference in MMP-2 labeling index between the A and G alleles.

Our study had certain limitations. First, because nasal polyps may recur several years after surgery, extended follow-up might be required. Second, the sizes of the samples used for comparing MMP-2 and MMP-9 labeling index and SNPs were small. We emphasize that this was an exploratory study and future confirmatory studies may be warranted. As we did not make any sample size calculation due to the lack of publications on this subject, the risk of type II error or an underpowered study is unavoidable. Third, the use of inferior turbinate mucosa as control might not be appropriate. However, we were not allowed to harvest sinus mucosa from patients without sinus lesion by our hospital IRB due to the ethical issue.

In conclusion, this study characterized the MMP-2 and MMP-9 expression in normal and inflamed upper airways. In normal nasal tissues, gelatinases in stromal and epithelial cells could be required for the maintenance of ECM homeostasis and submucosa gland remodeling. Nasal polyp exhibited higher levels of MMP-9 in glands and of MMP-2 in surface epithelium. Based on these results, we suggest that both MMP-2 and MMP-9 may play a part in the formation of nasal polyp and, more generally, in inflammatory airway remodeling. As therapeutic strategies with anti-MMP molecules are currently being developed [35], local control of the release and/or activation of MMPs may be another strategy for the treatment of nasal polyp, which is a common and disabling disease.

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