Rhinology

Expression profiles of MMP-9 and EMMPRIN in chronic rhinosinusitis with nasal polyps

Profili dell’espressione di MMP-9 e EMMPRIN nella rinosinusite cronica con poliposi nasali

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SUMMARY
Objective. Metalloproteinases (MMPs) are implicated in tissue remodeling in chronic rhinosinusitis with nasal polyps (CRSwNP). This study aimed to evaluate the expression profiles of MMP-9 and the extracellular matrix metalloproteinase inducer (EMMPRIN) in nasal polyps compared to healthy mucosa.

Methods. Tissue samples from 37 CRSwNP patients undergoing functional endoscopic sinus surgery and mucosal specimens from 12 healthy controls were obtained intra-operatively. MMP-9 and EMMPRIN mRNA levels were assessed by reverse transcription-polymerase chain reaction (RT-PCR) and their protein expression by Western blot analysis.

Results. MMP-9 mRNA expression levels were significantly elevated in CRSwNP compared to controls (p < 0.05). MMP-9 protein levels presented an increasing trend but without statistical significance (p > 0.05). No statistically significant difference in EMMPRIN mRNA and protein levels was identified.

Conclusions. Upregulation of MMP-9 in nasal polyps is evident and highlights its role in the pathogenesis of CRSwNP. The lack of concordance between MMP-9 mRNA and protein levels may be attributed to post-translational gene expression regulation. Although EMMPRIN expression was not significantly different between the two groups, its role remains to be elucidated. MMP-9 may be a valuable biomarker and treatment target in CRSwNP.

KEY WORDS: matrix metalloproteinases, nasal mucosa, nasal polyps, sinusitis

RIASSUNTO
Obiettivo. Le metalloproteinasi (MMPs) sono coinvolte nel rimodellamento tessutale proprio della rinosinusite cronica con poliposi (CRSwNP). Questo studio si prefigge lo scopo di confrontare l’espressione di MMP-9 e dell’induttore di metalloproteinasi della matrice extracellular (EMMPRIN) nei polipi nasali e nella mucosa sana.

Metodi. I campioni di tessuto sono stati ottenuti da 37 pazienti con CRSwNP sottoposti a chirurgia endoscopica funzionale dei seni paranasali e da 12 controlli sani. I livelli di mRNA di MMP-9 e EMMPRIN sono stati quantificati tramite reazione a catena della polimerasi inversa (RT-PCR) e la loro espressione proteica è stata analizzata tramite analisi di Western Blot.

Risultati. I livelli di espressione di mRNA MMP-9 erano significativamente elevati nei polipi nasali rispetto ai controlli (p < 0.05). La proteina MMP-9 era tendenzialmente più elevata nei polipi ma senza una differenza significativa (p > 0.05). Non è stata riscontrata alcuna differenza statisticamente significativa nell’espressione di mRNA e proteica di EMMPRIN.

Conclusioni. L’incremento di MMP-9 nei polipi nasali dimostra il suo ruolo nella patogenesi di CRSwNP. La discordanza tra l’espressione di mRNA e proteica di MMP-9 potrebbe essere attribuita ad aspetti di regolazione post-trascrizionali. Sebbene nessuna differenza di espressione di EMMPRIN sia stata dimostrata nei due gruppi, il ruolo di questa proteina deve essere ancora chiarito. MMP-9 potrebbe rappresentare un valido biomarker e target di trattamento in CRSwNP.

PAROLE CHIAVE: metalloproteinasi, mucosa nasale, polipi nasali, sinusite

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Introduction

Chronic rhinosinusitis (CRS) is a chronic multifactorial, inflammatory disease of the nose and paranasal sinuses affecting around 10% of the world’s population. As reported by the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2020), CRS is defined as sinonasal inflammation with a length of a minimum of 12 weeks, with two or more symptoms, one of which should be either nasal blockage, congestion, obstruction or anterior/posterior nasal discharge, accompanied by facial pain/pressure or decrease/loss of olfactory sensation.

CRS, being a highly heterogeneous disease, is classified depending on presence of polyps into CRS without (CRSs-NP) and CRS with nasal polyps (CRSwNP), which in turn also exhibits various endotypes. In the Western world, CRSwNP usually presents as Th2-based immune response with abundant tissue eosinophilic infiltrates, mast cells, and basophils and oedema. In Asian populations, it is characterised by a mixed T cell immune response and primarily neutrophilic infiltration. Eosinophilic CRSwNP presents with basement membrane thickening and goblet cell hyperplasia.

An imbalance in the dynamic process between extracellular matrix (ECM) accumulation and turnover, alongside thickening of the basement membrane and rupture, is postulated to be involved in tissue remodeling and the pathogenesis of nasal polyosis in CRSwNP. Interestingly, in the early stages of nasal polyp development, fibroblasts differentiated into myofibroblasts overproduce ECM proteins such as collagen type I, IV, VI, and fibronectin.

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147, is a cell-surface-glycosylated transmembrane protein, that belongs to the immunoglobulin superfamily. Its glycosylated form potentially induces the production of matrix metalloproteinases (MMPs), especially MMP-9 expression, and has a role in signaling pathways and transcellular communication.

MMP-9 (also referred to gelatinase B) is mainly implicated in type IV collagen degradation, and is an essential element of the ECM and basement membrane of the nasal epithelium. Except from its role in cell migration and proliferation, MMP-9 has also been found to regulate microvascular permeability and resultant oedema in the lower airways.

The aim of the present study was to assess the expression of MMP-9 and its inducer EMMPRIN in terms of mRNA and protein in patients with CRSwNP and compare them to healthy controls, hypothesising that they may play a role in tissue remodeling encountered in the disease.

Materials and methods

Study population and inclusion criteria

A total of 49 subjects (37 CRSwNP patients, aged between 18 and 72 years, average 46.3 years and 12 controls, average 45.2 years) were recruited in the period between September 2017 and January 2021.

Inclusion criteria for enrollment in the study group were age over 18 years, with CRSwNP scheduled to undergo functional endoscopic sinus surgery (FESS). The diagnosis of CRSwNP was based on standard preoperative computed tomography (CT) and endoscopy according to the diagnostic criteria set by the guidelines of the latest EPOS2020.

Exclusion criteria for the patient group were considered CRSsNP, allergic fungal rhinosinusitis, antrochoanal polyps, secondary forms of CRSwNP, neoplastic disease and cases with orbital, intracranial, and osseous extra-sinus complications of CRSwNP. In 2 cases originally included, an inverse papilloma, identified intra-operatively, was confirmed by pathology and these subjects were also excluded. Participants were selected randomly solely based on consecutive appointments for scheduled surgery.

The control group consisted of 12 participants who underwent septoplasty for nasal septum deviation without any other nasal pathology. Controls were selected arbitrarily based on the date of presentation for surgery.

Biological samples acquisition

Nasal polyp tissue samples were collected during FESS from 37 patients (30 male and 7 female) with CRSwNP, aged between 18 and 72 years. A portion of the excised tissue was sent for histopathological examination, while the remainder was stored at -80°C until processed. Nasal mucosal specimens from healthy controls were received from the inferior turbinate after separation from the underlying bone.

Real-time polymerase chain reaction (qRT-PCR) for mRNA quantification

RNA extraction was performed with the use of NucleoSpin RNA/protein kit (Macherey-Nagel) according to the manufacturer’s instructions. All samples were cryopreserved at -80°C and then lysed by adding 700 μl Lysis solution (RP1) and 3.5 μl of β-mercaptoethanol. After homogenisation, the homogenisation product was added in the column provided for disposal of particles not homogenised by centrifugation at 11,000 g for 1 minute. 350 μl of ethanol 70% were added to the repeatedly eluted suspension, until a clear suspension was received for total RNA extraction. The tissue elute was further centrifuged at 11,000 g for 1 min and stored at 4°C until RNA extraction was complete. 350 μl MDB...
were added and the elute was recentrifuged at 11,000 g for 1 minute. Total RNA was measured with an Multiskan Sky High Microplate Spectrophotometer (ThermoFischer). Measurements were performed at 230 nm and 280 nm to ensure RNA purity. The estimated ideal absorbance ratio at 260 nm versus 280 nm was 1.8. A 1.5% agarose gel in the presence of formaldehyde was used for the assessment of RNA quality, permitting visualisation of the 28S rRNA and 18S rRNA bands as markers of RNA integrity. Reverse transcription to complementary DNA (cDNA) of a total of 5 µg mRNA of total RNA per sample was performed by the reverse transcriptase enzyme Superscrip II (Invitrogen). Random nucleotide hexamers, serving as primers, were employed for cDNA synthesis, to warrant DNA synthesis from the whole mRNA. qPC reactions were performed with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems) using 50 ng cDNA as template. Reactions were set up in 96-well plates and executed on an MX3000P qPCR system (Agilent). The Ct values were analysed using the 2 ^ΔΔCT method after normalization against ACTB levels 5. The normalisation procedure was performed using one of the control samples (sample C1), which was analysed in each 96-well plate to ensure that all samples were equally compared to the same control. All reactions were performed in triplicates and the primer sequences are as follows: ACTB_F: AGCCGAGCATCCCCCAAGTT, ACTB_R: GGCGAC-GAAGGCTCATCATT, MMP9_F: CTTTGGACACGC-GACGAC, MMP9_R: CACCTGGTTCACAATCACTCCG, EMMPRIN_F: CTTCATCTACGAGAAAGCCCG, EMMPRIN_R: AATCTACGGGGTTGGTCTTCT.

Western blotting

Protein lysates were extracted using the NucleoSpin RNA/protein kit (Macherey-Nagel) according to the manufacturer’s instructions. 30-50 µg of total protein extracts were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Massachusetts, USA). Blocking in 5% (w/v) non-fat dry milk in TBS/0.05% Tween 20 was followed by incubation with primary antibodies overnight at 4°C and with goat anti-rabbit secondary HRP-conjugated antibody for 1h at room temperature. The Image Lab software (Bio-Rad, version 6.1) was employed to measure the band intensity of each experiment. For each set of experiments, normalisation of the protein levels against β-actin levels was performed, using one of the control samples (sample C1), which was separated in every SDS-PAGE confirming equal comparison of each sample to the same control. The following antibodies were utilised: anti-β-actin (#4967, Cell Signaling), anti-MMP9 (#13667, Cell Signaling), anti-EMMPRIN (#13287, Cell Signaling).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 software package. The unpaired parametric Student’s t-test was applied and data for all samples are presented as mean ± standard deviation (SD). Statistical significance was set at p < 0.05.

Results

Participant characteristics

The detailed demographic data and clinical characteristics of the participants are shown in Table I. No significant differences regarding age and gender were present between the study and control groups (p < 0.05). 14 of the CRS patients were smokers and 14 had concurrent asthma, while 5 of the controls were smokers and 2 had a history asthma.

mRNA levels of MMP-9 and EMMPRIN in CRSwNP

Expression profiles of MMP-9 in CRSwNP

Enhanced expression of the MMP-9 gene was detected in nasal polyp tissue samples (Figs. 1A-B). More specifically, MMP-9 was estimated to be 2.24-fold higher in the patient group (n = 37) compared to healthy controls (n = 12), a significant increase (p-value < 0.01) with a standard deviation ± 2.29. Smoking status did not correlate to a significant increase in MMP-9 expression in the CRSwNP group (Fig. 1C). The relative MMP-9 expression was estimated to be 2.96 (± 2.59) in smokers compared to 1.05 (± 0.52) in non-smokers (p-value < 0.05). Furthermore, no significant difference in MMP-9 expression was observed in patients with a history of asthma in comparison to those without asthma. The relative MMP-9 expression in asthma patients was 2.02 (± 1.49), while in non-asthmatic patients was 2.31 (± 2.68) (Fig. 1D).

mRNA levels of EMMPRIN in CRSwNP

Expression profiles of EMMPRIN in CRSwNP

EMMPRIN gene expression was found to be elevated in tissue samples from CRSwNP patients (Figs. 2A-B). This increase was estimated 0.95-fold higher (SD ± 0.60) for the nasal polyp samples (n = 37) compared with healthy mucosa (n = 12). Nevertheless, this increase was not significant. EMMPRIN expression did not differ significantly between smoking and non-smoking patients with CRSwNP (Fig. 2C). The relative EMMPRIN expression was estimated to be 1.07 (± 0.70) in smokers as opposed to 0.88 (± 0.53) in non-smokers (p-value < 0.05). Moreover, no significant increase in EMMPRIN expression was noticed in asthmatic CRSwNP patients compared to patients without asthma. The relative EMMPRIN expression in asthma patients was 0.95 (± 0.66), while in non-asthmatic patients was 0.96 (± 0.58) (Fig. 2D).
Protein expression of MMP-9 and EMMPRIN in CRSwNP

The results of Western blot analysis demonstrated a trend to increased expression of the protein levels of MMP-9 and EMMPRIN, but with no statistical significance (Figs. 3A-B). MMP-9 expression was estimated at 328.36% (± 258.07) for the CRSwNP group compared to 204.57% (± 85.17) for the healthy controls (n = 12). Additionally, in the study group, EMMPRIN expression was calculated at 225.94% (± 137.67) as opposed to 160.76% (± 51.05) in the control group. Smoking was not associated with a significant increase in MMP-9 and EMMPRIN expression in patients with CRSwNP. It should be noted, however, that a trend towards increased expression of MMP-9 was observed, in accordance with the mRNA levels (Fig. 3C). The relative MMP-9 expression was estimated to be 414.59% (± 260.58) in smokers as opposed to 269.80% (± 243.65) in non-smokers. The relative expression of EMMPRIN was 238.30% (± 91.26) in smokers with CRSwNP, while in non-smoking patients EMMPRIN expression was calculated to be 212.70% (± 92.36) in CRSwNP patients with asthma, while in non-asthmatic patients EMMPRIN expression was calculated to be 234.01% (± 160.60).

Discussion

In the present study, we aimed to evaluate the expression of MMP-9 and EMMPRIN in patients with CRSwNP. Our findings demonstrate that MMP-9 is highly expressed in polyp tissue samples from patients with chronic sinusal inflammation in terms of mRNA compared to healthy nasal mucosa and offer potentially significant insights into the role of MMP-9 in CRSwNP. Our results are overall in agreement with previous studies and support the involvement of MMP-9 in tissue remodeling observed in the disease. The protein levels of MMP-9 showed a slight upregulation but with no statistical significance, a finding that is not in accordance with the mRNA levels of MMP-9 gene, where a statistically significant upregulation was observed. A possible reason for this finding is the small number of experimental samples. Moreover, a post-transcriptional gene expression regulation program could take place involving different classes of non-coding RNAs (ncRNAs), such as miRNAs. Their involvement in the regulation of gene ex-
pression is well studied and involves targeting of specific mRNAs and repression of translation. Several miRNAs have been reported to possibly regulate the expression levels of *MMP-9*. It was recently reported that a miR29b3p/MMP9 axis is involved in the pathogenesis of CRSwNPs. miR-29b-3p has been found to directly target *MMP-9* mRNA and that miR29b3p expression is moderately positively correlated with the expression of MMP9 in CRSwNPs. Other miRNAs that directly or indirectly affect MMP-9 expression include let-7e, miR-129, miR-335, miR-211, miR-491-5p and miR-1253. Moreover, novel classes of ncRNAs that can regulate gene expression are tRNA-derived fragments (tRFs) and long ncRNAs, whose role in the pathogenesis of CRSwNP remains to be elucidated.

![Graphs showing mRNA levels of MMP-9 in CRSwNP](image)

**Figure 1.** mRNA levels of *MMP-9* in CRSwNP. (A-B) relative expression of *MMP-9* in controls and patients. *MMP-9* expression was significantly increased in the patient group; (C) comparison of the relative expression of *MMP-9* between smoking and non-smoking CRSwNP patients showed upregulation of *MMP-9* among the smoking group; (D) Asthma was not associated with a significant increase in *MMP-9* expression in CRSwNP patients.

CRSwNP: chronic rhinosinusitis with nasal polyps.
MMP-9 exhibits an important role in modifying extracellular components and participates in normal embryonic development, as well as healing through tissue remodeling. It is mainly produced by inflammatory cells such as macrophages, neutrophils, fibroblasts and airway epithelial cells. While the regulation of its expression remains multifaceted and not fully clarified, gene transcription, proenzyme activation and inhibition by specific and nonspecific inhibitors are the major points implicated.

Due to its potent role in tissue remodeling, MMP-9 has been evaluated as a possible biomarker or therapeutic target in CRSwNP. However, these studies have identified equal distribution of either non-significant or increased MMP-9 levels in patients with CRSwNP. Topographic distribution of either non-significant or increased MMP-9 levels in patients with CRSwNP.
studies of MMP-9 have identified the protein in different tissues, with increased staining in the epithelial layer. In the majority of those studies, the authors agree that nasal polyp tissue exhibits increased MMP-9 expression in the superficial epithelium, endothelial cells, adenoid cells and extracellular matrix in comparison to healthy controls.

Similar to the findings of our study, increased expression of MMP-9 has been shown in nasal polyp tissue samples with immunohistochemistry and RT-PCR. An upregulation of MMP-9 has been demonstrated in both polyp tissue (evaluated with RT-PCR) as well as in CRSwNP patient serum (assessed with ELISA) and correlated this increase to the severity of the disease.

MMP-9 expression has been found elevated in recurrent and non-recurrent CRSwNP in comparison to healthy controls, without a significant difference in terms of expression between the two patient groups, presumably due to different pathogenetic mechanisms responsible for recurrent disease, outside of MMPs. Moreover, higher amounts of MMP-9 in nasal secretions and connective tissue have been associated with worse healing following sinus surgery, while it also appears to contribute to the development of osteitis in CRS.

EMMPRIN constitutes an upstream inducer of MMPs, capable of modulating MMP expression, composed of two domains one transmembrane and a short cytoplasmic one. Although first identified in tumour cells where it stimulated interstitial collagenase (MMP-1) production by regional fibroblasts, overexpression of EMMPRIN has also been documented in diverse inflammatory lower airway disease including chronic obstructive pulmonary disease (COPD).

Figure 3. Protein levels of MMP-9 and EMMPRIN in CRSwNP. (A) Western blots of MMP-9 and EMMPRIN in controls (C1-C12) and patients (P1-P37). (B) relative % protein levels of MMP-9 and EMMPRIN in controls and patients; (C) comparison of relative expression of MMP-9 and EMMPRIN between smoking and non-smoking CRSwNP patients showed no significant difference between the two groups but a trend of the MMP9 protein to increase on smokers; (D) asthma was not associated with a significant increase in MMP-9 and EMMPRIN expression in CRSwNP patients. CRSwNP: Chronic rhinosinusitis with nasal polyps.
and smoking \cite{21} with a subsequent increase in MMP expression. One study has previously investigated EMMPRIN in nasal polyps and found increased EMMPRIN levels, suggesting increased ECM degradation in the disease \cite{22}. In a similar study in COPD, blockage of EMMPRIN has led to a decrease in MMP-9 \cite{20}. Moreover, patients with COPD had higher levels of EMMPRIN expression in bronchoalveolar lavage (BAL) compared to healthy smokers and non-smokers. In the present study, no alterations were observed regarding the mRNA and protein levels of EMMPRIN in CRSwNP patients compared to healthy controls. This finding though does not exclude the involvement of EMMPRIN in CRSwNP. It is known that EMMPRIN induces the production of MMP-9 through a post-translational modification that involves glycosylation. Moreover, EMMPRIN has been indicated to act as a key regulatory molecule in various signaling routes and cell to cell interactions \cite{3}. Taking into consideration the aforementioned, the possible involvement of EMMPRIN in the pathogenesis and progression of CRSwNP could involve other mechanisms and not upregulation of its protein. Smoking has been correlated with an increase in MMP-9 in CRSwNP patients. In a study including smoking and non-smoking CRSwNP patients, PCR and Western Blot showed elevated MMP-9 expression \cite{23}. However, in the present study, cigarette smoking was not associated with MMP-9 and EMMPRIN upregulation.

CRS and asthma are currently being regarded as different manifestations of the spectrum of an inflammatory disease of a ‘united airway’ considering the frequent comorbidity of asthma and CRS, as well as the presence of comparable pathologic processes such edema and airway remodeling. The levels of MMP-9 have been correlated to the severity of asthma and lung function decrease. According to Katainen et al. patients with asthma, CRSwNP, or both tend to have lower MMP-9 levels in nasal secretions, but higher MMP-9 serum levels compared to healthy controls \cite{24}. According to our results, the expression of MMP-9 in tissue samples was found increased in both CRSwNP subgroups, independently of the presence of asthma, without a significant difference between the two. This finding may reflect enhanced expression of MMP-9 due to the underlying CRS irrespective of the presence of asthma. Our study has certain limitations, most important of which is the relatively small sample size, preventing solid deductions especially as far as correlation parameters are concerned. Moreover, another potential limitation is the lack of correlation of our findings on MMP9 and EMMPRIN expression with the specific disease endotype. Lastly, due to specimen collection in patients undergoing FESS for CRSwNP, the nasal polyp tissue received and assessed may not be indicative of the early stages of polypogenesis and may not be indicative of the changes in MMP expression in the initial phases of the disease.

Conclusions

Our findings confirm the contribution of MMP-9 in the occurrence and progression of CRSwNP, as is manifested by its increased expression and as suggested by previous research. Larger studies may help assess the proteolytic spectrum of CRSwNP and the implication and clinical significance of EMMPRIN in the pathophysiological pathway of MMP, as well as the regulatory mechanisms involved in the disease.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

SL: data collection, preparation and final review of the manuscript; GD: data collection, review of the manuscript; KG: data processing, preparation of the manuscript; FT: literature review and collection, preparation of the manuscript; KG: data processing, review of the manuscript; CS: design of the research, supervision of data analysis, review of the manuscript; VD: design, implementation and supervision of the research and final review of the manuscript.

Ethical consideration

This study was approved by the Institutional Ethics Committee (Research and Ethics Committee of the University Hospital of Patras) (approval number/protocol number 403/01.08.2017).

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association’s Declaration of Helsinki. Written informed consent was obtained from each participant/patient for study participation and data publication.

References

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