รายงานวิจัยฉบับสมบูรณ์

โครงการ : การศึกษายีนแกมมาโกลบินในริดสีดวงจมูกที่มีและไม่มีโรคภูมิแพ้ร่วมด้วย

โดย รองศาสตราจารย์แพทย์หญิงสุพินดา แสงพานิชย์และคณะ

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Expressions of *mammaglobins A* and *B* are not different between nasal polyps with and without allergic rhinitis

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Key words: Nasal polyps, Allergic rhinitis, *Mammaglobin*

Running title: *Mammaglobins*' expressions in nasal polyps

Abstract

Background: Nasal polyposis is a chronic disease of nose and sinuses. Its actual causes remain unclear. Mammaglobins have been implicated in the pathogenesis of nasal polyps. However, their association with the occurrence of nasal polyps in the presence of allergic rhinitis is still inconclusive. The aim of this study was to compare the expression levels of *mammaglobin A* and *B* between the nasal polyps with allergic rhinitis and without allergic rhinitis.

Methods: 31 patients with bilateral nasal polyposis underwent skin prick test to specific aeroallergens. Nasal polyp tissues were obtained from all patients and divided into 2 groups as nasal polyps with allergic rhinitis and nasal polyps without allergic rhinitis depending on the skin prick tests' result. All polyp tissues were analyzed for the level of *mammaglobin A* and *mammaglobin B* by using using real-time quantitative polymerase chain reaction technique (RTQ-PCR).

Results: *Mammaglobin A* expressed in only one nasal polyp tissue (mean expression 0.023) out of 16 different samples from patients having nasal polyps with allergic rhinitis (1/16). There was no expression of *mammaglobin A* in tissues from the group of nasal polyps without allergic rhinitis (0/15). Expression of *mammaglobin B* was detected in all nasal polyp tissues from both groups. The mean expression of *mammaglobin B* was not significantly different between nasal polyps with allergic rhinitis (0.059) and nasal polyps without allergic rhinitis (0.133).

Conclusions: Expressions of *mammaglobin A* and *B* are not different between nasal polyps with and without allergic rhinitis. From previous studies implicating mammaglobins in the pathogenesis of nasal polyps, we proposed that they do so independent of an underlying allergic rhinitis.

Introduction

Nasal polyposis is a chronic inflammatory disease of the nasal cavities. A population-based study in Europe found the prevalence of nasal polyposis to be 4.3% similar to the prevalence of physician-diagnosed asthma.[1] It is also common in the United States affecting 2% to 5% of the population.[2] A study in Thai patients with allergic rhinitis revealed a 4.5% incidence of nasal polyps.[3] Patients with nasal polyposis often present with progressive nasal congestion, hyposmia, rhinorrhea, and facial pain.[4] If left untreated, the nasal polyps can grow so large that they expand the nasal bones and broaden the nasal bridge.[5] Treatment with intranasal corticosteroids remains the first line of treatment due to their ability to reduce symptoms and size of nasal polyps. However, most patients with mechanical obstruction caused by nasal polyps do not response to medical treatment requiring endoscopic sinus surgery and polypectomy. In addition, recurrence of nasal polyps after surgical removal is frequently seen.[6] Understanding of its molecular pathogenesis, therefore, may lead to a better preventive measure and a more effective treatment. Allergy, infection and genetic susceptibility have been postulated to play a role in its pathogenesis.[7] Although its definite causes have not been clearly identified, most studies support the role of inflammation and local immunologic imbalance in the development of nasal polyps.[8]

With the recent advance in genetic techniques, genes involved in pathogenesis of nasal polyps have been started to unveil.[9]Of these, *mammaglobins A* and *B* are the two that have been implicated. Mammaglobin is a 10-kd glycoprotein that is distantly related to a family of proteins that includes rat estramustine-binding protein and human Clara cell protein (CC10/uteroglobin). The function of the mammaglobin protein is unknown. However, its related family members are small epithelial

secretory proteins that can either modulate inflammatory process or bind steroid ligands. In addition, regrowth of nasal polyps can be prevented by using corticosteroids. These might give some clues to mammaglobin function.

The first study implicating *mammaglobin* in nasal polyposis was performed by Fritz et. al. in 2003. They found that the most upregulated gene in nasal polyps of patients with allergic rhinitis compared to nasal mucosa of patients with allergic rhinitis but without polyps was *mammaglobin 1* (or *mammaglobin A*).[10] Another study observed similar tendency showing that *mammaglobin* (A or B) was modestly upregulated in nasal polyps comparing to normal sinus tissues.[9] On the contrary, Benson et. al. demonstrated a lower, though not statistically significant, mean expression levels of *mammaglobin B* in nasal polyps compared to healthy nasal mucosa.[11] Due to the discrepancies of expression levels of *mammaglobins* in nasal polyps, further studies of their association with the disease are required.

We hypothesize that the involvement of mammaglobins in the pathogenesis nasal polyps may be dependent on the underlying allergic rhinitis occurring in patients with.nasal polyps. We, therefore, performed the study aiming to compare the expression level of *mammaglobin A* and *mammaglobin B* between nasal polyps with and without allergic rhinitis.

Material & Methods

Subjects

We included all patients presented with bilateral nasal polyposis who entered Department of Otolaryngology, King Chulalongkorn Memorial hospital and were willing to participate in the study. Written informed content was obtained from patients prior to their recruitment. The Ethic Committee of Faculty of Medicine,

Chulalongkorn University approved the study. Treatment with antihistamines and intranasal corticosteroids was discontinued for at least 2 weeks before undergoing skin prick test to specific aeroallergens and nasal polyp biopsy. The aeroallergen extracts included house dust, dust mites, cockroaches, cats, dogs, feathers, Bermuda grasses, Johnson grasses and molds. A reaction was considered positive if the wheal was 3 millimeters or larger in mean diameter with surrounding erythema. The patients who had positive skin test responses were diagnosed as nasal polyps with allergic rhinitis. All patients underwent nasal polyp biopsies under local anesthetic and topical decongestant using standard nasal endoscopy with 4-mm rigid endoscope. Polyp tissues were divided into 2 groups as polyps with allergic rhinitis and polyps without allergic rhinitis. All tissues were analyzed for the level of *mammaglobin* expression by using real-time quantitative polymerase chain reaction technique (RTQ-PCR).

Analysis of mammaglobin RNA level

Total RNA was isolated from the biopsied tissues using the QIAamp RNA Blood Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA was reverse transcribed into complementary DNA (cDNA) using ImProm-II™ reverse transcriptase (Promega, Madison, Wisconsin, USA), according to the company recommendations. All RNA samples were stored at -70ºC before use.

Quantitative RT-PCR was performed using Roter-Gene™ 6000 (Corbett Robotics Inc, Mortlake, NSW, Australia) according to the manufacturer's instructions. The TaqMan primer/hybridization probe real-time PCR approach was used to assay the expression level of *mammaglobin A*. The primers for all mRNA assays were intron-spanning. The sequences of the amplification primers and TaqMan binding probes for *mammaglobin A* and *GAPDH* are listed in Table 1. *GAPDH* was used as a control for normalization.. *Mammaglobin A* RNA level was determined by

Biotools QuantiMix Easy Probes Kit (Biotools, Madrid, Spain). The PCR reactions were set up according to the manufacturer's instructions in a reaction volume of 10 µl. Each reaction contained 1×quantiprobe; 1×Rox dye; 0.25 µM of each primer; and 0.125 µM fluorescent probe. Cycling times and temperatures were as follows: initial denaturation was carried out for 7 minutes at 95ºC, followed by 35 cycles of denaturation at 94ºC for 15 s and combined primer annealing/extension at 60ºC for 25 s. For *mammaglobin B* expression, SYBR Green I (10,000×concentration in DMSO; Molecular probes, Eugene, OR, USA) was used as the detection format. The sequences of the amplification primers for *mammaglobin B* and *GAPDH* are listed in Table 1. Amplification was carried out in a total volume of 10 µl containing 1×SYBR Green I, PCR buffer (<<<KCl, Tris-HCl, $MgCl_2$), 0.15 µM of each primer, 0.15 mM dNTPs, 0.5 U Taq DNA polymerase (Fermentas, Burlington, ON, Canada), 1.5 mM $MgCl_2$, 1×Rox and 1 µl of cDNA. After initial denaturation at 95ºC for 5 minutes. The reactions were cycled 45 times under the following parameters: 95ºC for 20 s, 59ºC for 15 s, 72ºC for 20 s. A nontemplate control was run with every assay and each sample was assayed in triplicate.

A standard curve from the amplification data for each primer was generated using a dilution series of cDNA as templates. The expression levels of *mammaglobins* were normalized to the expression of *GAPDH*. All data were analyzed using Q-gene softwares, a widely-used program for relative quantitation available at http://www.biotechniques.com/softlib/qgene.html.

Statistical analysis

The variables were the level of mammaglobins expression in nasal polyps tissue, sex, age and number of subject expressing *mammaglobins*. The differences of the levels of expressions of *mammaglobin A* and *mammaglobin B* in polyp tissues

between nasal polyps with allergic rhinitis and nasal polyps without allergic rhinitis were compared using independent-sample T test. Because the data were normal distribution, parametric statistics were used for analysis and the levels of expressions were shown as mean and range. Data for age, sex and number of subject expressing *mammaglobins* were analyzed using Chi-square tests to compare between nasal polyps with allergic rhinitis and nasal polyps without allergic rhinitis group.

Results

31 patients with bilateral nasal polyps were recruited. The skin prick test results were positive in 16 patients (6 females, aged 18 to 67 years, average 45.9 years) and negative in 15 patients (6 females, aged 21 to 71 years, average 40.9 years) (Table 2). Polyp tissues from the patients were analyzed for the expression levels of *mammaglobin A* and *mammaglobin B* by using real-time PCR technique. *GAPDH* was used as a control for normalization. *Mammaglobin A* expressed in one nasal polyp tissue (mean expression 0.023) out of 16 different samples from all patients having nasal polyps with allergic rhinitis (1/16). There was no expression of *mammaglobin A* in tissues from the group of nasal polyps without allergic rhinitis (0/15). Expression of *mammaglobin B* was detected in all nasal polyp tissues from both groups. The mean normalized expression of *mammaglobin B* in tissues from nasal polyps with allergic rhinitis was 0.059 (range 0.0002 to 0.343), while the mean normalized expression of *mammaglobin B* was 0.133 (range 0.003-0.628) in tissues from nasal polyps without allergic rhinitis. The expression levels between both groups were not significantly different.

Discussion

Mammaglobins have been implicated in the pathogenesis of nasal polyps but their roles are still inconclusive. Some studies found that *mammaglobin A* was upregulated[10], while another showed that it was not changed[9]. Interestingly, *mammaglobin B* was found to be downregulated[11]. The discrepancy may relate to the presence of allergic rhinitis. We, therefore, compared the expressions of *mammaglobins A* and *B* in nasal polyps with and without allergic rhinitis.

We found that only one out of 16 samples in the group of nasal polyps with allergic rhinitis expressed *mammaglobin A*, while none of the 15 samples in the group of polyps without allergic rhinitis expressed it. This finding was different from the study by Fritz et al., which detected mammaglobin A protein in three of the five samples from nasal mucosa of patients with polyps but none in controls without nasal polyps.[6] The difference is unlikely from a technical problem of our study in quantitative assay by RTQ-PCR as we were able to detect its expression in one subject (see Figure 1, picture of RT-PCR and the standard curve). Instead, this could be partly due to differences in pathogenesis among different ethnics and geographic distributions. In addition, it could be from the difference in tissue samplings between two studies. In our study, we performed biopsies at the polyps whereas in Fritz's study, the biopsies were done at the origin of the polyps. Nonetheless, our study supported that of Benson et al., which did not implicate *mammaglobin A* in the pathogenesis of nasal polyps.

We found that *mammaglobin B* was expressed in all samples we studied. However, the expression levels were not significantly different between nasal polyps with and without allergic rhinitis. Previous studies using DNA microarray analyses of various cells and tissues revealed that *mammaglobin B* and *uteroglobin* were only expressed in nasal polyps and nasal mucosa. In addition, Benson et al. demonstrated

the mean expression levels of *mammaglobin B* in nasal polyp were lower but did not significantly differ when comparing with healthy nasal mucosa .[11]

In conclusion, expressions of *mammaglobins A* and *B* are not different between nasal polyps with and without allergic rhinitis. From previous studies implicating mammaglobins in the pathogenesis of nasal polyps, we propose that allergic rhinitis does not contribute to the development of nasal polyps through the action of mammaglobins.

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Table 1: Primers and probe sequences

Targets	Primers	Sequences (5'-3')
Mammaglobin A	SCGB2A2-F79	CTCATGCTGGCGGCCCTCTC
	SCGB2A2-P106	(FAM)-TGCTACGCAGGCTCTGGCTGCCC-(BHQ1)
	SCGB2A2-R236	ATGGCATTTGTAGTGGCATTGTC
Mammaglobin B	SCGB2A1-F261	TCCTCAACCAGTCACATAGAAC
	SCGB2A1-R380	CCCTCTGAGCCAAACGCCTT
GAPDH	GAPDH-F85	GTGAAGGTCGGAGTCAACGG
	GAPDH-P121	(HEX)-CGCCTGGTCACCAGGGCTGC-(BHQ1)
	GAPDH-R191	TCAATGAAGGGGTCATTGATGG
GAPDH	GAPDH-F6	GAAGGTGAAGGTCGGAGTC
	GAPDH-R231	GAAGATGGTGATGGGATTTC

FAM: 6-carboxyfluorescein, BHQ1: Black Hole Quencher, HEX: hexachloro-6-carboxy-fluorescein

Table 2 Characteristics and expressions of *Mammaglobins A* and *B* in polyps with and without allergic rhinitis

	Polyps with allergic rhinitis	Polyps without allergic rhinitis	P value
Sex (n) F	6	6	0.589
M	10	9	
Age (Mean (SD), range, years)	45.9 (13.7), 18-67	40.9 (18.2), 21-71	0.389
Number of subject expressing *Mammaglobin A*	1/16	0/15	NS
Number of subject expressing *Mammaglobin B*	16/16	15/15	NS
Level of *mammaglobin B* expression (Mean (SD), range)	0.059 (0.089), 0.005-0.343	0.133 (0.215), 0.0002-0.628	0.228

Figure legend

Fig 1. Quantitation of *mammaglobin A* mRNA-positive cells by real-time PCR in the Rotor Gene System. Left panel. Logarithmic plot of fluorescence signal during amplification. Right panel. Standard curves of RTQ-PCR of serial RNA dilutions extracted from four samples A. *Mammaglobin A,* B. *Mammaglobin B,* C. *GAPDH* used to normalize *Mammaglobin A*, D. *GAPDH* used to normalize *Mammaglobin B*

Executive Summary

1. ความสำคัญและที่มาของปัญหา

เนื่องจากริดสีดวงจมูกเป็นโรคที่พบประมาณ 4-5% ของประชากร และการเกิดโรคจะทำให้ผู้ป่วยมีอาการคัดจมูก ไม่ได้กลิ่น และมีไซนัสอักเสบเรื้อรังแทรกซ้อน ปัจจุบันพยาธิกำเนิดโรคยังไม่ทราบแน่ชัด อย่างไรก็ตามการศึกษาที่ผ่านมาพบว่าปัจจัยที่สำคัญที่มีผลต่อการเกิดโรคคือ ภาวะภูมิแพ้และการอักเสบเรื้อรัง

การศึกษาบทบาทของยีนที่น่าจะมีผลต่อการเกิดโรคริดสีดวงจมูกเริ่มมีการศึกษามาไม่นานมานี้ โดยมีการเปรียบเทียบ gene expression ในผู้ป่วยโรคจมูกอักเสบจากภูมิแพ้ที่มีริดสีดวงจมูก พบว่า *mammaglobin* ในเนื้อเยื่อริดสีดวงมากกว่าเยื่อบุผิวจมูกที่ไม่มีริดสีดวงจมูกถึง 12 เท่า ดังนั้น *mammaglobin* gene น่าจะเป็นปัจจัยสำคัญต่อการเกิดริดสีดวงจมูกในผู้ป่วยโรคภูมิแพ้ แต่ในปัจจุบันยังไม่มีการศึกษาเปรียบเทียบ *mammaglobin* gene expression ในริดสีดวงจมูกในผู้ป่วยที่มีโรคภูมิแพ้กับผู้ป่วยริดสีดวงจมูกที่ไม่มีโรคภูมิแพ้ การได้ข้อมูลของ *mammaglobin* gene expression ในริดสีดวงจมูกทั้งสองกลุ่ม จะทำให้สามารถอธิบายความสำคัญของ *mammaglobin* ต่อการเกิดโรคริดสีดวงจมูก และความสัมพันธ์กับโรคภูมิแพ้

2. วัตถุประสงค์

เพื่อตรวจหาระดับของ *mammaglobin* gene expression ในเนื้อเยื่อริดสีดวงจมูกที่มีภูมิแพ้และไม่มีภูมิแพ้ร่วมด้วย

3. ระเบียบวิธีวิจัย

ทำการศึกษาวิเคราะห์ดูระดับของ *mammaglobin* gene ในเนื้อเยื่อริดสีดวงจมูกในผู้ป่วยริดสีดวงจมูก เปรียบเทียบกับเนื้อเยื่อปกติของคนปกติ และดูความสัมพันธ์เปรียบเทียบระหว่างผู้ป่วยที่มีโรคภูมิแพ้ร่วมด้วยและผู้ที่ไม่มีโรคภูมิแพ้ โดยให้งดการใช้ยา corticosteroids ก่อนตัดชิ้นเนื้อเป็นเวลา 2 สัปดาห์ โดยแบ่งเนื้อเยื่อจากผู้ป่วยออกเป็น 2 กลุ่ม คือ กลุ่มที่ 1 เนื้อเยื่อริดสีดวงจมูกในผู้ป่วยที่มีจมูกอักเสบจากภูมิแพ้ร่วมด้วย กลุ่มที่ 2 เนื้อเยื่อริดสีดวงจมูกในผู้ป่วยที่ไม่มีจมูกอักเสบจากภูมิแพ้ร่วม ทำการวัดระดับของ *mammaglobin* gene expression โดยวิธี quantitative PCR ผลที่ได้จะนำมาวิเคราะห์ทางสถิติเปรียบเทียบระหว่างผู้ป่วยทั้ง 2 กลุ่ม

4. แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วงรวม 24 เดือน

Activities	**Month**											
	2	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**	**24**
Subjects recruitment	/	/	/	/	/	/	/	/	/			
***mammaglobin* analysis**	/	/	/	/	/	/	/	/	/	/		
Analysis of data											/	
Conclusion / reporting												/

5. ผลงาน/หัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติในปี 2550-2551

Expressions of *Mammaglobins A* and *B* are not different between nasal polyps with and without allergic rhinitis

ในวารสาร **American Journal of Rhinology**

ชื่อโครงการ	วัตถุประสงค์หลัก	งบประมาณ	ตัวชี้วัด	ระยะเวลา
Mammaglobin expression in patients with nasal polyposis with or without allergic rhinitis	ดูระดับของ *mammaglobin* expressionในผู้ป่วยริดสีดวงจมูกเพื่ออธิบายพยาธิกำเนิดของโรคและความสัมพันธ์ในผู้ป่วยที่มีหรือไม่มีจมูกอักเสบจากภูมิแพ้		*Mammaglobin* expression	2 ปี