

Original article

Effects of antiallergic herbal agents on cystic fibrosis transmembrane conductance regulator in nasal mucosal epithelia of allergic rhinitis rabbits

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Keywords: *transmembrane conductance regulator protein; antiallergic herbal agents; allergic rhinitis*

Background It has been found that the expression of cystic fibrosis transmembrane conductance regulator (CFTR) is closely related to allergic rhinitis (AR). In the previous study, we have demonstrated that antiallergic herbal agents (AHA) can obviously inhibit the allergic reaction of AR. The aim of this study was to explore the expression of CFTR and the effects of AHA on CFTR to improve the allergic reaction of AR.

Methods An animal model of an AR rabbit was established using ovalbumin (OVA). The rhinitis rabbits were randomly assigned to three groups: AHA treating group (AHATG), modeling group (MG) and healthy controlling group (HCG). The expressions of CFTR protein were examined by immunohistochemical method. The mucosal epithelial cells of all the rabbits were primarily cultured with tissue culture method *in vitro* and treated with or without glibenclamide for 24 hours. The levels of monocyte chemotactic factor-1(MCP-1) and RANTES protein in supernatants of culture were measured by ELISA, and the expressions of CFTR mRNA were detected by real-time PCR.

Results The expressions of CFTR mRNA and protein greatly increased in mucosal epithelial cells of MG. The protein concentrations of MCP-1, RANTES in culture supernatants of MG were significantly higher than those in the other two groups ($P < 0.01$), and they reached much higher level than those at the start points in the MG ($P < 0.05$) and were significantly different compared with those in the AHATG after being cultured for 24 hours ($P < 0.01$). CFTR mRNA in MG + glibenclamide were much lower than those in MG ($P < 0.05$). RANTES and CFTR mRNA treated with glibenclamide in AHATG were significantly lower than those in the AHATG ($P < 0.01$). Minimal changes in the secretions of MCP-1 in the epithelial cells were detected between AHATG and AHATG + glibenclamide ($P > 0.05$).

Conclusions AHA can inhibit the secretions of CFTR, RANTES and MCP-1 in mucosal epithelia and improve inflammatory reaction of AR. CFTR may play an important role in the secretion of RANTES and mucosal inflammatory response in AR. Glibenclamide can inhibit the CFTR secretion in mucosal epithelial cells, in particular during AR process. These effects of glibenclamide on secretion of RANTES can be effectively strengthened by AHA.

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Allergic rhinitis (AR) is characterized by a complicated interaction between inflammatory mediators but ultimately is triggered by IgE-mediated response to the extrinsic protein. It involves the release of mediators and recruitment of leukocytes or inflammatory cells into mucosal sites. The proper homeostasis of liquid that lines the surface of the respiratory tract is vital for mucociliary clearance, which plays an important role in host defense. It has been found that cystic fibrosis transmembrane conductance regulator (CFTR) was closely related to mucosal edema and inflammation in AR.¹ High expression of CFTR in nasal mucosa has been observed in nasal polyps.^{2,3} Some studies had linked the activity of ion channels to immune response in nasal mucosal epithelial cells of AR.^{4,5} Antiallergic herbal agents (AHA) were antiallergic herbal products.⁶ In the previous study, we have showed that AHA can obviously inhibit allergic medium and release the allergic reaction in the nasal mucosal epithelial cells of AR rabbits.⁷⁻⁹ The aim of this study was to explore the expression of CFTR and the effects of AHA on CFTR to improve the allergic reaction of AR.

METHODS

Materials

New Zealand white rabbits (25 males, 8–12 weeks of age, 4.0–4.5 kg) were purchased from the Animal Laboratory Department of Medicine, Beijing University, China. AHA was produced in Tianjin Institute of Integrated Traditional Chinese and Western Medicine (z20010035), China. Ovalbumin (OVA) (grade V) and glibenclamide

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aluminum hydroxide powder were bought from Sigma, America. The rabbit monocyte chemotactic factor-1 (MCP-1) and RANTES ELISA kit were purchased from Uscnlife, America. Glibenclamide (1×10^{-4} mol/L) was obtained from Sigma. Anti-rabbit CFTR mAb and chromogenic enzyme substrate DAB were provided by Uscnlife. Avidin-biotin complex (ABC) was bought from DAKO, Denmark. RNeasy mini and real-time PCR kit was purchased from Qiagen, Germany. Reverse transcription system was obtained from Promega, America.

Preparation of AHA

Prescriptions of AHA component mainly were breviscapus, wind, changium and atractylodes macrocephala. Among them, atractylodes macrocephala and wind were extracted by 70% ethanol. Ethanol was decompressed and absorbed by alcohol liquid. The cream of the agents at the concentration of 1:20 was prepared. Breviscapus and changium were extracted from volatile oil and concentrated from the cream of the agents at the concentration of 1:20. Furthermore, both creams were mixed and diluted by distillation. The AHA was produced at the concentration of 2 g/ml. All processes of production were followed by good laboratory practice (GLP) criteria.

Establishment of a rabbit model and AHA application

OVA was used to sensitize and challenge the rabbits. The OVA sensitized solution was prepared using 30 mg OVA as Ag emulsified in 3 g of alum and mixed with 100 ml saline. Rabbits were randomly classified into 3 groups including AHA treating group (AHATG), modeling group (MG) and healthy control group (HCG). The AHATG and MG were sensitized by intraperitoneal injection of sensitized solution 5 ml on days 0, 2, 4 and 6 following the protocols. The HCG was injected with the same dose of saline at the same day. After passive-cutaneous-anaphylaxis (PCA) was tested and OVA IgE Ab titer was increased, the rabbit model of AR was made. Then OVA sensitized solution was administered by nosedrop as a single dose challenge in the AHATG and MG by 10 μ l/side each day, 7 days. The HCG was dropped with the same dose of saline. At the same time, the AHATG was fed with AHA 5 ml/d, 7 days. The HCG and MG were administered the same dose of saline. Animals were sacrificed on day 14. Mucosal epithelial tissue was resected for further examination.

Primary culture of mucosal epithelial cell and glibenclamide treatment

Small 2 mm³ pieces of mucosal epithelial tissue resected from rabbit models of all groups were placed into 1 ml of ice Dulbecco's modified Eagle's medium (DMEM) immediately after resection. Tissue was washed with phosphate buffered solution (PBS) (contained 500 U/ml penicillin, 500 pg/ml streptomycin) and incubated in PBS (contained 1% EDTA and 0.05% collagenase type II) at 37°C for 1 hour. The epithelial pieces were pipetted vigorously for 5 minutes, and the resulting cell

suspension was transferred to a centrifuge tube and centrifuged at $300 \times g$ for 5 minutes. Mucosal epithelial cells were harvested and washed. They were seeded into 96-well plates at a density of 1×10^4 cells per well in a DMEM culture medium containing 20% fetal bovine serum (FBS) for 24 hours treated with or without glibenclamide and referred with the following groups: (1) AHATG; (2) MG + glibenclamide; (3) AHATG + glibenclamide; (4) HCG + glibenclamide; (5) MG; (6) HCG. After 24 hours, supernatant was collected for further test. MCP-1 and RANTES in the supernatant were analyzed by ELISA method. CFTR mRNA was detected by real-time PCR.

Real-time PCR detection of CFTR mRNA

Total RNA was isolated using the RNeasy mini kit. Total RNA from mucosal epithelial cells was extracted with TRIzol reagent following the manufacturer's instructions. RNA samples were quantified using an UltrospecTM 1100 Pro UV-Visible spectrophotometer and diluted to the same concentration. Reverse transcription was done on 1 μ g of total RNA using the reverse transcription system with random primers and oligo dT at the ratio of nine to one. The following primers were used for quantitative real-time PCR: (F) 5'-AGCATTGCTGATTGCACAG-3' and (R) 5'-GACCAGAAATCGTTGGCATT-3' for CFTR, (F) 5'-CTGCCCTCTTCACAGTTTC-3' and (R) 5'-TCATGACAAGGTAGGGCTCC-3' for reduced glyceraldehydes-phosphate dehydrogenase (GAPDH). Real-time PCR was carried out on a CFX96 real-time PCR detection system. All RT and PCR were performed in triplicate with and without RT as controls. Cycle threshold (C(t)) values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ analysis.

Immunohistochemistry

The secretion of CFTR in mucosal epithelial tissue sections was analyzed by immunostaining using an anti-rabbit CFTR mAb. Briefly, sections were captured on slides and dewaxed. The Ag retrieval was performed by microwave pressure cooking in citric acid buffer. Before staining the sections, endogenous peroxidase was blocked with 0.1% bovine serum albumin (BSA). The slides were then incubated overnight with the anti-rabbit CFTR Ab, washed with triethanolamine-buffered saline solution (TBS). The immunodetection was performed with biotinylated anti-mouse secondary Ab (30 minutes), followed by ABC (30 minutes), and diaminobenzidine chromogen as substrate. Sections were counterstained with Meyer's hematoxylin and mounted.

RANTES and MCP-1 ELISA

The protein concentrations of MCP-1 and RANTES in nasal mucosa and primary culture of mucosal epithelial cell supernatant were assayed by ELISA. Briefly, the tissue homogenate and supernatant were added to the coated (anti-MCP-1 and RANTES antibody) and blocked 96-well plates. Then, biotinylated anti-MCP-1 and RANTES antibody, streptavidin-horseradish peroxidase

(HRP) and substrate (TMB) were added sequentially per kit according to the manufacturer's protocols. The OD values were obtained by a microtiter autoreader at 450 nm. The concentrations of cytokine were calculated by comparison with the standard curve.

Statistical analysis

Data were expressed as mean $\pm$ standard error (SE). One way analysis of variance (ANOVA) and *q* test were used in data analysis. Statistical analysis was carried out using SPSS13.0. $P < 0.05$ was considered statistically significant.

RESULTS

High-level expressions of CFTR mRNA in mucosal epithelia

Real-time PCR results showed that the mRNA of CFTR was highly expressed in the rabbit mucosal epithelial cells in the MG, while little in the HCG. The expressions of CFTR mRNA level were decreased in the AHATG compared with that in the MG (Figure 1).

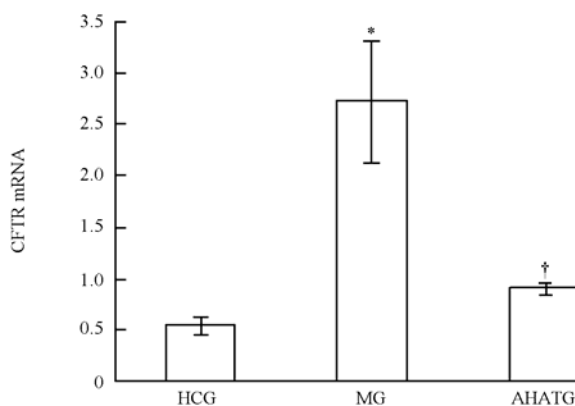


Figure 1. Relative band density of CFTR mRNA in mucosal epithelial cells of the AHATG, MG and HCG by real-time PCR detection. CFTR mRNA was significantly increased in the MG compared with that in the HCG (* $P < 0.01$) and it was significantly decreased in the AHATG compared with that in the MG († $P < 0.01$).

The expressions of CFTR protein in mucosal epithelia

Immunohistochemical staining of rabbit mucosal epithelia was used to visualize the presence of CFTR protein in mucosal epithelia of allergic rhinitis. Immunohistochemistry displayed that CFTR protein was strongly expressed in the MG. The expression of CFTR protein was detected little in the HCG. The expression of CFTR protein decreased after the AHA treatment. The expression of CFTR protein of mucosal epithelia in the MG was obvious in nasal epithelium and in submucosal glands. Particularly, CFTR protein was seen to be located in epithelial lamina and periglands. Epithelial interstitial edema and basement membrane fracture were obviously observed. After the treatment with AHA, the above performance in the MG was significantly improved (Figure 2).

Effects of AHA on mucosal epithelial cell secretion

Then expressions of RANTES, MCP-1 protein and CFTR mRNA in the MG were significantly higher than those expressed in the AHATG ($P < 0.01$). After the epithelial cells were cultured for 24 hours, the expressions of RANTES, MCP-1 protein reached much higher level in the MG than those at the start points ($P < 0.05$) and were significantly different compared with those in the AHATG ($P < 0.01$) (Figure 3).

Effects of glibenclamide on cell secretion and functions of AHA

After the epithelial cells were treated with glibenclamide for 24 hours, the level of CFTR mRNA in the MG+glibenclamide was lower than those expressed in the MG ($P < 0.01$). The levels of RANTES and MCP-1 in the MG+glibenclamide were not significantly different compared with those in the MG ($P > 0.05$). The expressions of CFTR mRNA and RANTES showed significant difference between the AHATG and AHATG + glibenclamide ($P < 0.01$). Minimal changes in the secretion of MCP-1 in the epithelial cells were detected between the AHATG and AHATG + glibenclamide ($P > 0.05$) (Figure 3).

DISCUSSION

Mucosal edema is the most typical and prominent pathological change in allergic rhinitis. In surface epithelium, absorption of NaCl and water is the significant physiological transport process. The rate of Na^+ absorption is enhanced by CFTR deletion and reduced by CFTR stimulation under normal conditions. The structure of the submucosal glands suggests an important role of serous cell liquid secretion in the elaboration of the secretory product. Substantial evidence has suggested that the process of regulated insertion-retrieval also applies to CFTR. Riordan et al¹⁰ found that cystic fibrosis (CF) was caused by mutations in the CFTR gene. At the airway surface, deletion of CFTR causes hyperabsorption of sodium chloride and reduction in the periciliary salt and water content, which impairs mucociliary clearance and leads to a progressive cycle of infection and inflammation. Bucholtz et al² observed that the abnormal distribution of CFTR in nasal epithelium can cause nasal polyps. Lin et al³ showed that CFTR protein was over-expressed in the tissue of nasal polyps. Several measurement methods have been employed to examine these issues, and studies have been conducted in human airway epithelia both *in vitro* and *in vivo*. Gu et al¹¹ found the alveolar fluid clearance decreased obviously after alveolar epithelium was treated with glibenclamide *in vitro*. Schwiebert et al¹² demonstrated that CFTR modulated migration process of leukocytes into the cystic fibrosis lung and the expression of chemokines, including interleukin-8, MCP-1 and RANTES. It was possible that a loss of CFTR activity in CF epithelial cells may alter the activity of transcription

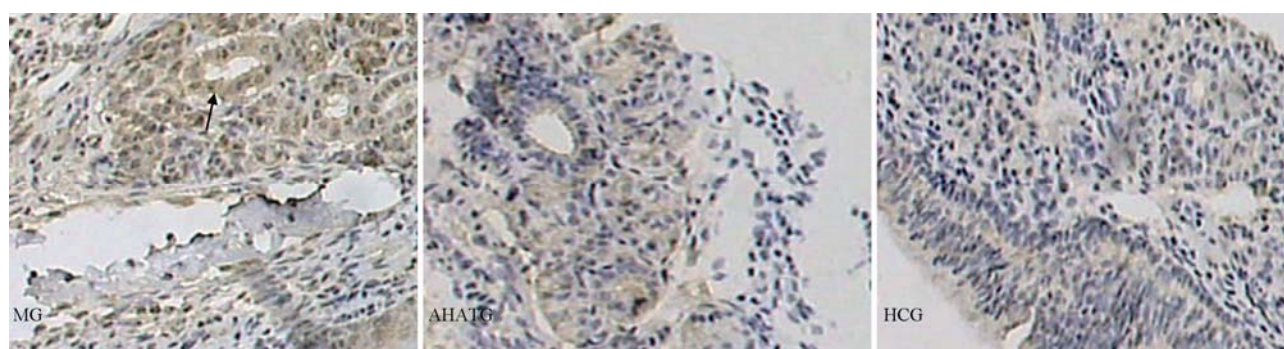


Figure 2. Expressions of CFTR in nasal mucosa by immunohistochemistry (IHC, original magnification, $\times 200$). Positive staining (arrow) is showed in epithelia and submucosal glands of the MG. Less and weaker staining expressed in submucosal glands of the HCG and of AR samples after treatment with AHA.

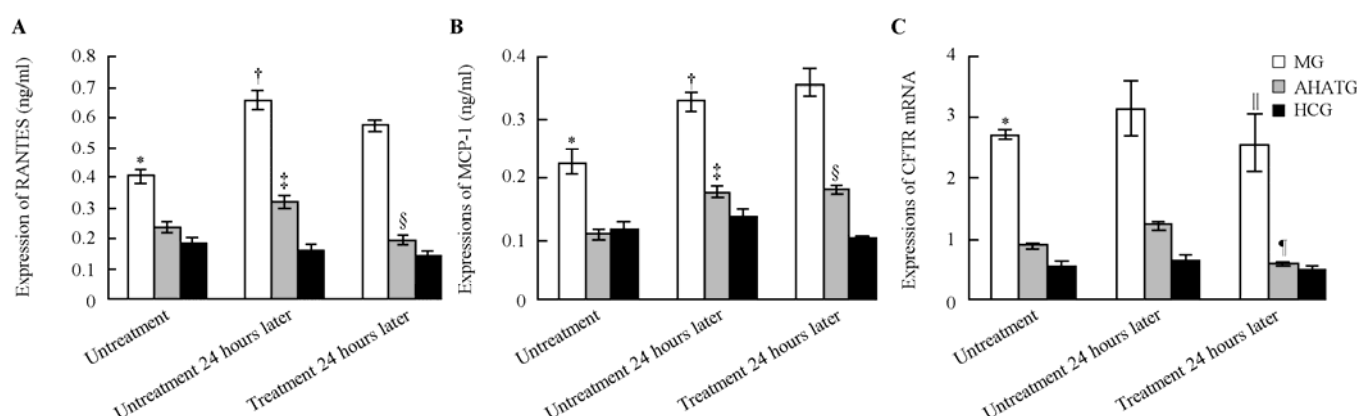


Figure 3. Expressions of RANTES (A), MCP-1 (B) protein and CFTR mRNA (C) in the MG, AHATG and HCG treated with or without glibenclamide. The RANTES, MCP-1 protein and CFTR mRNA in the MG were significantly higher than those expressed in the AHATG and HCG ($^*P < 0.01$). After 24 hours, the expressions of RANTES and MCP-1 protein in the MG were greatly increased than those at the start points ($^*P < 0.05$) and significantly different compared with those in the AHATG ($^*P < 0.01$). **A:** After 24 hours treatment, expressions of RANTES in the MG + glibenclamide were not significantly different compared with that expressed in the MG ($P > 0.05$). But they were significantly decreased in the AHATG + glibenclamide compared with those in the AHATG ($^*P < 0.01$). **B:** There were no significant differences showing in secretion of MCP-1 treated with glibenclamide for 24 hours among the AHATG, the AHATG + glibenclamide and MG + glibenclamide ($P > 0.05$). **C:** Expressions of CFTR mRNA in the MG + glibenclamide were significantly decreased compared with those in the untreated MG ($^*P < 0.05$). They showed significant decrease in the AHATG + glibenclamide compared with those in the AHATG ($^*P < 0.01$) after treatment with glibenclamide for 24 hours.

factors such as NF- κ B and the subsequent induction of RANTES gene expression. Several studies have linked the activity of ion channels to immune responses *in vivo*.¹³

Our study demonstrated that both CFTR protein and mRNA expression could be detected in nasal epithelium and in submucosal glands of AR. Simultaneously, the pathological changes of epithelial interstitial edema and basement membrane fracture occurred. RANTES is one of the most widely studied chemokines linked to allergic diseases.^{14,15} It is chemotactic for T cells, human eosinophils and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. RANTES also activates process in eosinophils. MCP-1 may participate in the pathogenesis of allergic rhinitis, thus contributes to proinflammatory cells attraction and mediator release,^{16,17} which plays an important role during the late phase of the allergic reaction. In our study, we demonstrated that the release of RANTES and MCP-1 increased in nasal epithelial cells of allergic rhinitis. After

we block CFTR in the epithelial cells with glibenclamide, the protein expression level of CFTR was lower than untreated groups. These effects of glibenclamide on secretion of CFTR can be effectively strengthened by AHA. In the condition of being treated with AHA, the expression of RANTES can be intervened effectively by glibenclamide, the inhibitor of CFTR. Minimal changes of the protein secretion levels of MCP-1 in the epithelial cells were detected after being treated with glibenclamide. Consequently, CFTR may have effect on immune reaction in allergic rhinitis. We further demonstrated that CFTR probably can influence the secretion of RANTES in AR, but not MCP-1. AHA would play an important role in regulating CFTR function in AR. It needs to further invest the mechanism of the effects of CFTR on RANTES in AR.

AHA was antiallergic herbal products. Prescription of AHA was source from traditional prescription of guomin-jian.⁶ It has been used to treat the allergic rhinitis effectively in clinic. In the previous study, we have

verified that AHA clearly inhibited allergic medium and relieved the allergic reaction in nasal mucosal epithelia of allergic rhinitis rabbits.⁷⁻⁹ In the present study, we further found the possible mechanism of AHA therapeutic effects on immune responses in allergic rhinitis. The reduction of CFTR level is related to AHA treatment. Moreover, we demonstrated that AHA can enhance the glibenclamide function in blocking the secretion of CFTR and RANTES from epithelia of AR. AHA may react on the multiple mechanisms in the epithelial secretion in AR directly or indirectly. The prescription components of AHA were breviscapus, wind, changium and atractylodes macrocephala. According to the theory of traditional Chinese medicine, atractylodes macrocephala and wind have good performance that enhance immune function, breviscapus and changium can reduce exudation in nasal mucosal epithelia in AR. Compound Chinese medicine may have advantages over Western medicine. Some potent pharmacological compounds have been reported to inhibit the chloride channel in allergic rhinitis and asthma.¹⁸ This is the new field.^{19,20} In this study, the results of regulatory function of AHA to CFTR, inflammatory mediators and improvement of inflammatory reaction in AR provide a basis for further research in mechanism of Chinese medicine in AR treatment.

REFERENCES

- Duszyk M. CFTR and lysozyme secretion in human airway epithelial cells. *Pflügers Arch Eur J Physiol* 2001; 443 Suppl: s45-s49.
- Bucholtz GA, Ejercitko VS, Burmester JK. The cystic fibrosis conductance regulator gene exon sequence is normal in a patient with edematous eosinophilic nasal polyps. *Am J Rhinol* 1999; 13: 221-223.
- Lin S, Lin G, Huang J, Jiang Z. Expressions of cystic fibrosis transmembrane conductance regulator in epithelial cells of nasal polyps and normal turbinate mucosae. *Chin J Otorhinolaryngol Head Neck Surg (Chin)* 2005; 19: 19-22.
- Rosenberg MF, Kamis AB, Aleksandrov LA, Ford RC, Riordan JR. Purification and crystallization of the cystic fibrosis transmembrane conductance regulator (CFTR). *J Biol Chem* 2004; 279: 39051-39057.
- Ostedgaard LS, Rokhlina T, Karp PH, Lashmit P, Afione S, Schmidt M, et al. A shortened adeno-associated virus expression cassette for CFTR gene transfer to cystic fibrosis airway epithelia. *Proc Natl Acad Sci USA* 2005; 102: 2952-2957.
- Zhang EH. *Manual of clinical medicine*. Beijing: People's Medical Publishing House; 2002.
- Shen XH, Chen J, Lin WS. Experimental study on the pharmacological effect of Biminling in a way of various grouping components on iNOS in nasal mucosa with allergic rhinitis. *Chin Otorhinolaryngol J Integ Med (Chin)* 2008; 16: 166-167.
- Shen XH, Chen J, Li WS. Effect of Biminling on the apoptotic activity of nasal epithelia among guinea pigs with experimental allergic rhinitis. *Chin Otorhinolaryngol J Integ Med (Chin)* 2007; 15: 81-83.
- Shen XH, Chen J, Lin WS. An experimental study of two-ways regulation of buqigu biao on the apoptosis in nasal mucosa of allergic rhinitis. *Tianjin Med J (Chin)* 2007; 35: 44-45.
- Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 1989; 245: 1066-1073.
- Gu X, Li SZ, Zhao Li, Tsutomu S. Effects of CFTR in alveolar fluid clearance of rats *in vitro*. *Chin J Tuberc Respir Dis (Chin)* 2006; 29: 57-59.
- Schwiebert LM, Estell K, Propst SM. Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *Am J Physiol Cell Physiol* 1999; 276: 700-710.
- Schwiebert EM, Morales MM, Devidas S, Egan ME, Guggino WB. Chloride channel and chloride conductance regulator domains of CFTR, the cystic fibrosis transmembrane conductance regulator. *Proc Natl Acad Sci USA* 1998; 95: 2674-2679.
- Chihara J. The role of chemokines such as RANTES in allergic disease. *Rinsho Byori* 1998; 18: 324-328.
- Garrelds IM, Veld Cde G, Wijk RG, Zijlstra FJ. Nasal hyperreactivity and inflammation in allergic rhinitis. *Mediators Inflamm* 1996; 5: 79-94.
- Zhu K, Shen Q, Ulrich M, Zheng M. Human monocyte-derived dendritic cells expressing both chemotactic cytokines IL-8, MCP-1, RANTES and their receptors, and their selective migration to these chemokines. *Chin Med J* 2000; 113: 1124-1128.
- Casaleand TB, Amin BV. Allergic rhinitis/asthma interrelationships. *Clin Rev Allergy Immunol* 2001; 21: 27-49.
- Gu ZY, Gu RJ. Drug therapy of allergic rhinitis. *Chin J Otorhinolaryngol (Chin)* 2000; 35: 73-75.
- Rogers DF. Airway hypersecretion in allergic rhinitis and asthma: new pharmacotherapy. *Curr Allergy Asthma Rep* 2003; 3: 238-248.
- Li J, Wang HY, Zhang CG, Sun BQ, Zhong NS. Links between allergic rhinitis and asthma. *Chin Med J* 2006; 119: 676-683.

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