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Effect of RNA interference therapy on the mice eosinophils CCR3 gene and granule protein in the murine model of allergic rhinitis

Xin-Hua Zhu[1], Bing Liao[2], Ke Liu[1], Yue-Hui Liu[1*]

[1]*Department of Otorhinolaryngology Head and Neck Surgery, the Second Affiliated Hospital of Nanchang University, Nanchang 330006, China*
[2]*Department of Head and Neck Surgery, the Tumor Hospital of Jiangxi Province, Nanchang 330006, China*

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ABSTRACT

Objective: To observe the clinical manifestations of allergic rhinitis mice and the expression changes of the eosinophils CCR3 and the granule protein mRNA in the bone marrow, peripheral blood and nasal lavage fluid. **Methods:** Twenty-four BALB/c mice were randomly divided into the control group, PBS therapy group, siRNA therapy group and the CCR3 siRNA therapy group (n=6). Allergic rhinitis model were sensitized and stimulated by ovalbunfin, and CCR3 siRNA therapy group were administered with CCR3 transnasally before stimulated. The levels of the eosinophils CCR3, MBP, ECP and EPO in bone marrow, peripheral blood and nasal lavage fluid were detected by RT-PCR. **Results:** Compared to the control group and CCR3 siRNA therapy group, the nasal mucosa of the PBS therapy group and siRNA therapy group developed epithalaxy, goblet cells hyperplasia, squamous epithelium metaplasia, epithelium necrosis, lamina propria and submucosa gland hyperplasia, vasodilatation, tissue edema, and the characterized eosinophil infiltration. RT-PCR indicated that the CCR3 mRNA, MBP , ECP and EPO expression in bone marrow, peripheral blood and nasal lavage fluid of the CCR3 siRNA therapy group was lower than the PBS therapy group and siRNA therapy group (P<0.05). **Conclusions:** The RNA interference therapy to CCR3 by local administration pernasal can suppress the process of the development, migration and invasion of the allergic rhinitis eosinophil, thus can reduce the effect of eosinophils and then reduce the inflammation effect of the allergic rhinitis. It may be a new treatment for respiratory tract allergic inflammation.

1. Introduction

Eosinophils (EOS) infiltration is an important pathological features of allergic diseases. In recent years, the study suggests that it is an important factor in the pathogenesis of allergy that EOS migration and activation to the local tissue. With the application of molecular biology techniques, the chemokine receptor 3 (CCR3) receptors were considered to be the EOS selective receptor which with a strong inducing chemotaxis effect. The traditional drug therapy has a poor specificity and the treatment is ineffective. RNA interference has the advantage of specific and targeting *etc*, is regulated by a large number of gene expression of a small amount of dsRNA. Currently the reports of the effect of inhibiting CCR3 expression on the allergic rhinitis is still rare. In this study, we established a mouse model of allergic rhinitis and used synthesized interfering RNA *in vitro*, and then observed the changes of mRNA of the EOS CCR3 and eosinophil granule proteins-major basic protein (MBP), cationic protein (ECP), peroxidase (EPO) expression in mice. This stody is to explore the feasibility of the treatment of RNA interference (RNAi) for allergic rhinitis.

*Corresponding author: Yue-Hui Liu, Department of Otorhinolaryngology Head and Neck Surgery, the Second Affiliated Hospital of Nanchang University, Nanchang 330006, China.
Tel: 15979022986
E-mail: liuyuehuiclark@21cn.com

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2. Materials and methods

2.1. Animals and reagents

Twenty four male BALB/c mice from 6 to 8 weeks, weight 20-30 g were purchased from animal and science of

medicine department of Nanchang University. Ovalbumin were purchased from Sigma Co., JM109 competent cells, a small amount of plasmid extraction kit, Trans2K Plus Ⅱ DNA Marker were purchased from Beijing Trans-Gen Biotech Co., DNA Primers Synthesis, DNA sequencing were purchased from Invitrogen Corporation of USA. Restriction endonuclease *Eco*R Ⅰ, restriction endonuclease *Hin*d Ⅲ, restriction endonuclease *Mlu* Ⅰ, restriction endonuclease *Bam*H Ⅰ, restriction endonuclease *Xho* Ⅰ, T4 DNA Ligase were purchased from NEB Inc. Qiagen large-scale plasmid extraction kit was purchased from Qiagen, Germany.

2.2. Construction of CCR3siRNA expression plasmid vector

CCR3 full-length clone (NM_009914. 4) was used as a template to amplify the coding frame sequence. Primer was designed and synthesized by BioWit-Technologies. shRNA sequences: shRNA1: GGTTGTGTTGATCCTCATAAA shRNA2: GCTGACAATTGACAGATACCT; shRNA3: GCAGCATTGCCTGAATTTATC; shRNA4: GACCACACCCTATGAATATGA. RNAi target sequence was designed and the target sequence was synthesized into Oligo DNA, annealing for forming the double-stranded DNA, and generated shRNA lentiviral vectors with pLVX-shRNA2-m vector which were digested by *Mlu*I, *Sac* Ⅰ, *Eco*R Ⅰ, *Hin*d Ⅲ, *Bam*H Ⅰ and *Xho* Ⅰ. Applied shRNA Lentiviral vector-transfected 293T cells and EOS cells, determined virus titer. Q-PCR identified the downward effect of CCR3 gene in EOS. Successfully constructed lentiviral vector plasmid (pLVX-ShRNA2-mCCR3-1 +2 +3 +4).

2.3. Animal grouping and sensitized model establishment.

In order to avoid the interference caused by external allergen, all the animals were kept in the laboratory of the department of animal science of medical college of Nanchang University, free diet, feeding by circadian rhythm. Twenty four clean grade BALB/c mice were randomly divided into 4 groups (n=6), one cage for each. Group one was the control group given normal saline. Group 2 was the PBS group given PBS. Group 3 was the siRNA group, given siRNA lentiviral vector. Group 4 was the CCR3 siRNA treatment group, given CCR3 siRNA. 8 μL treatment fluid were dropped into the nasal cavity on day 0 and day 14, respectively, twice a day. The ovalbumin (OVA)/aluminum hydroxide [AL $(OH)_3$] mixture [containing 10 μg OVA and 4 mg AL $(OH)_3$] was injected into the intraperitoneal for sensitization on day 2 and day 16, twice a day. 600 μg/mL OVA were dropped intranasal to stimulate allergic reactions from day 21 to day 27. The normal control group were replaced by the same dose of normal saline. The specimens of the mice were collected after 24 h of the administration.

2.4. Specimen collection and determination

Mice were anesthetized by intraperitoneal injection of 5% sodium pentobarbital. After anesthesia, the mice were fixed. Then 1 mL syringe needle was put into the nasal cavity about 2 cm bilateral. 2 mL PBS buffer was used to lavage the nasal and recovered the liquid. Pressing the eyeball at the mouse inner canthus, tear the retrobulbar tissues and collect blood. Exposure the nasal and obtained the bilateral nasal mucosa. The mucosa was fixed in 4% paraformaldehyde solution, embedded in paraffin, made into 4 μm sections for HE staining. The bone marrow, peripheral blood, nasal lavage fluid were stored at -80 ℃ refrigerator, detected by CCR3, EPO, MBP and ECP. Performed real-time quantitative PCR for the CCR3, EPO, MBP, ECP, and housekeeping gene (β-actin). Reaction conditions: 95 ℃ denaturation 2 min, 40 cycles, 94 ℃ denaturation 20 s, 49.5 ℃ (CCR3), 55.2 ℃ (EPO), 52 ℃ (MBP), 50.9 ℃ (ECP), annealed 20 s, elongation at 72 ℃ for 30 s, plate-reading at 74 ℃. PCR products were detected by agarose gel electrophoresis. Standardized eNOS, ICAM-1, Tenascin-C and PGF2a mRNA absorbance values with the internal reference β-actin optical density value, and obtained the relative content of CCR3, EPO, MBP and ECP mRNA expression.

The sequence of the CCR3, EPO, MBP and ECP and β-actin were as follow: CCR3 upstream primer: 5′CCA GAG GGT GAA GAA GAC 3′, downstream primer: 5′ACC AGG AAG AAA CGG AAT 3′. EPO upstream primer: 5′TGG AAA GAG GCG TAA TGG 3′, downstream primer: 5′AAG GAT GTA AGT GCG TTG AT 3′. MBP upstream primer: 5′GTT CCC AAC ACC AAG ACA 3′, downstream primer: 5′TCC CAT CCA TCC AGC AAA 3′. ECP upstream primer: 5′ATC ACT CAT CTG CCA AGC 3′, downstream primer: 5′CAG GGT TCA CAA GGG ACT 3′. β-actin primer: 5′GAG ACC TTC AAC ACC CCA GC 3′, downstream primer: 5′ATG TCA CGC ACG ATT TCC C 3′. Applicate the transmission ultraviolet analyzer for photograph, and then use a laser optical density image scanner to scan. The β-actin was used as an internal reference for semi-quantitative image analysis.

2.5. Result determination

Grading: (1) 0: no sneezing, no scratching nose, no secretion in nasal cavity; (2) 1 score: times of sneeze <4, mild scratching nose, the secretions in anterior nostril; (3) 2 scores: times of sneeze: 4-10, frequent scratching nose, nasal secretions more than anterior nostril; (4) 3 scores: times of sneeze> 11, continuous scratching nose, rubbing, face covered with nasal secretions. Superimposed quantization method was used to record the score. Nasal symptoms of 1-4 groups were observed after 30 minutes of the stimulation, the behavior score was calculated according to this rating system (> 5 is an successful animal model).

2.6. Statistical analysis

The data were analyzed by SPSS 19. 0 statistics software, and the measurement data were expressed as mean± SD values. Normal test and homogeneity of variance in each group was performed. The data which not normally

distributed were converted to normally distributed data by natural logarithm. t-test was applied in the comparison between two groups. $P<0.05$ was considered as statistical significance.

3. Results

3.1. Behavioral observation

The average score of the three groups was greater than 5 means successful model. The mice in the normal control group and CCR3 siRNA group had mild scratching nose and less sneezing, total score was <5. That means the allergic rhinitis animal model can be successfully established by this method, and the application of CCR3 siRNA treatment can effectively control the occurrence of allergic rhinitis.

3.2. Nasal histological changes

Compared group 1 with group 2 and group 3, the mucosa epithelium were shed and the goblet cell hyperplasia was obvious and coexsisted with EOS and other inflammatory cell infiltration, also with mucosa layer thickening and the dilatation and congestion of lamina propria, and tissue edema, glandular hyperplasia, hypertrophy strong secretion. That is comply with the pathological features of allergic rhinitis (Figure 1).

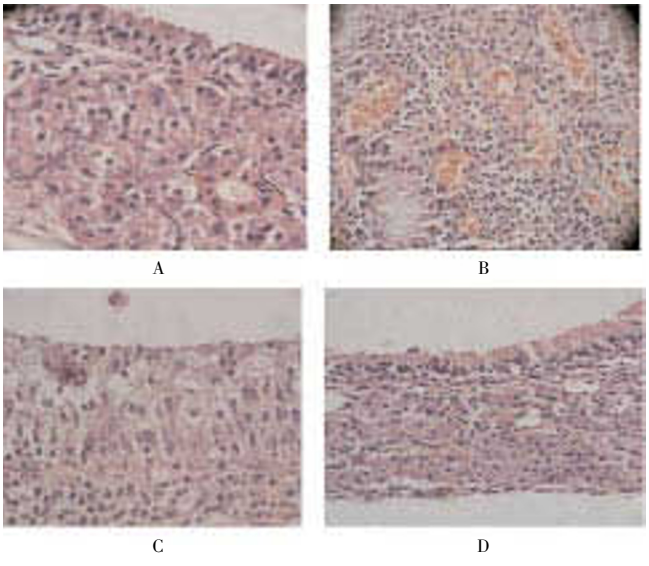

Figure 1. HE staining of nasal mucosa.
A. Group 1: Nasal epithelial cells arranged neatly, no inflammatory cell infiltration and no expansion of the gland in the lamina propria (HE ×200).
B. Group 2: Nasal lamina propria vascular dilatation and congestion, edema, with the infiltration EOS and other inflammatory cell, glandular hyperplasia, hypertrophy, strong secretion (HE × 200).
C. Group 3. Nasal epithelium, obviously infiltration of inflammatory cells of the goblet cell hyperplasia, thickened mucous layer, tissue edema (HE × 200).
D. Group 4: Nasal mucociliary surface layer is relatively complete, no thickening of the mucous layer (HE × 200).

3.3. RT-PCR test results

In this study, we detected bone marrow, peripheral blood and nasal lavage fluid of fresh specimens from 2-4 groups of the mice by RT-PCR, and found that after the treatment of pLVX-ShRNA2-mCCR3-1 +2 +3 +4 for topical nasal, the difference has statistically significant ($P<0.05$) (Figures 2, 3, Table 1).

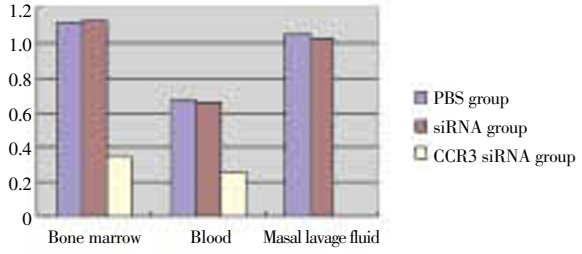

Figure 2. CCR3 mRNA expression of bone marrow, blood, nasal lavage fluid of mice in each group.

The relative gray value of CCR3 mRNA of each sample in CCR3 siRNA treatment group were as follow: Bone marrow (0.340 3±0.022 5), blood (0.253 4±0.061 6), nasal lavage fluid (0.00±0.00), which were significantly lower than the relative gray value of CCR3 mRNA of each sample in the PBS control group and the siRNA group ($P<0.05$).

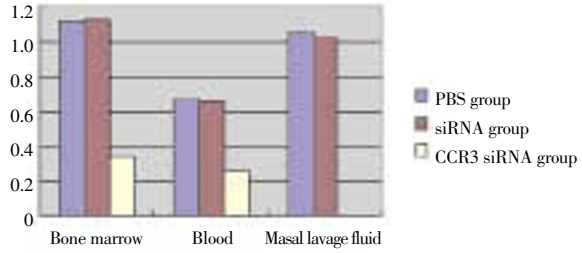

Figure 3. Eosinophil MBP mRNA expression of bone marrow, blood, nasal lavage fluid of mice in each group.

The MBP mRNA relative gray value of each sample in the CCR3 siRNA group were as follow: Bone marrow (0.324 0±0.024 5), blood (0.015 3±0.012 8), nasal lavage fluid (0.00±0.00) which were significantly lower than the MBP mRNA relative gray value of the PBS group and the siRNA group ($P<0.05$).

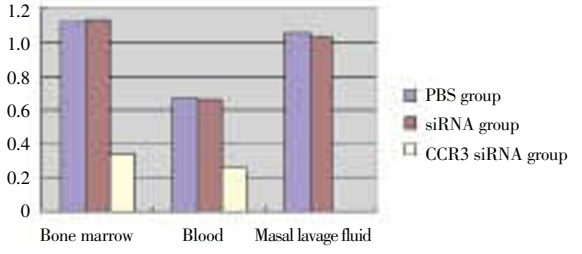

Figure 4. ECP mRNA expression of bone marrow, blood, nasal lavage fluid of mice in each group.

The ECP mRNA relative gray value of each sample in the CCR3 siRNA treatment group were as follow: bone marrow (0.4936±0.0115), blood (0.1405±0.0260), nasal lavage fluid (0.00±0.00) which were significantly lower than the ECP mRNA relative gray value of the PBS-treated group and the siRNA group (P<0.05).

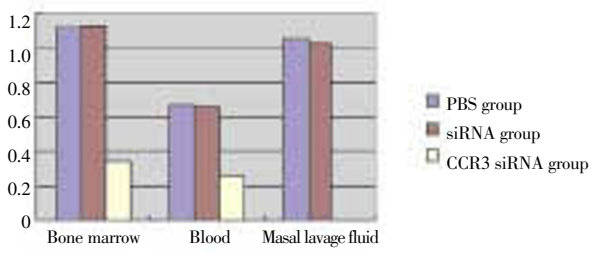

Figure 5. EPO mRNA expression of bone marrow, blood, nasal lavage fluid of mice in each group.

The EPO mRNA relative gray value of each sample in the CCR3 siRNA treatment group were as follow: bone marrow (0.1595±0.0599), blood (0.0842±0.1108), nasal lavage fluid (0.00±0.00) which were significantly lower than the EPO mRNA relative gray-value of the PBS-treated group and the siRNA group (P<0.05).

Table 1
CCR3, MBP, ECP, EPO mRNA relative gray values of the bone marrow, the blood and the nasal lavage fluid in three groups ($\bar{x}$±sd).

Groups	Bone marrow	Blood	Nasal lavage fluid
PBS-treated control group			
CCR3	1.1155±0.0480	0.6708±0.0265	1.0489±0.0132
MBP	0.8529±0.0650	0.7837±0.0712	1.0331±0.0469
ECP	0.9771±0.0379	0.7002±0.0416	0.9908±0.0553
EPO	0.4851±0.0546	0.6973±0.1334	1.0502±0.0410
siRNA-treated control group			
CCR3	1.1223±0.0461	0.6579±0.0147	1.0286±0.0260
MBP	0.9143±0.0362	0.6976±0.0681	1.0864±0.0531
ECP	0.9245±0.0467	0.7456±0.2971	1.0576±0.0647
EPO	0.5617±0.0746	0.7789±0.0171	0.9764±0.0847
CCR3 siRNA treatment group			
CCR3	0.3403±0.0225	0.2534±0.0616	0.000±0.000
MBP	0.3240±0.0245	0.0153±0.0128	0.000±0.000
ECP	0.4936±0.0115	0.1405±0.0260	0.000±0.000
EPO	0.1595±0.0599	0.0842±0.1108	0.000±0.000

Note: Compared with the PBS-treated control group and the siRNA treatment group, P<0.05 (t test), the difference was not statistically significant. Compared PBS-treated control group with the siRNA-treated control group, P>0.05(t test), the difference was not statistically significant.

4. Discussion

Allergic rhinitis belongs to type Ⅰ allergic reaction, which means after atopic individuals exposed to the allergens, the IgE-mediated media (mainly histamine) released and a variety of immune cells and cytokines involved in the nasal mucosa then caused this non-infectious inflammatory diseases. A large number of EOS infiltration in tissues is an important feature of allergic diseases, and EOS play a role of antigen-presenting cells in allergic airway disease[1,2]. EOS can release cytotoxic granule proteins, reactive oxygen species and lipid mediators, which can cause tissue damage, vascular leakage, mucus secretion and airway contraction[3]. It has been reported[3-5] that the pathogenesis of allergic diseases may be as follows: in the process of allergen challenge, the bone marrow can generate specific inflammatory cells (EOS progenitor cells), which can reach to the local tissue by peripheral blood circulation and then led to the local inflammation.

CCR3 is a G protein coupled receptor generated by transmembrane, primarily expressed in the EOS. Eosinophil activation chemokine (Eotaxin) belong to class CC chemokines, which combined with the CCR3 and induce eosinophil migrate to specific tissues, causing local tissue inflammation. Eotaxins can also play an important role in situ hematopoiesis of the EOS in sites of inflammation by CCR3 receptors[4-9]. RNAi is a mechanism which can make target gene silencing after specific transcription, dsRNA can cause the degradation of the homologous mRNA and lead to the undetectable expression of target gene. It can blocking the specific gene expression efficiently. Therefore, the use of RNAi technology for screening genome have been studied extensively. That is, design corresponding dsRNA for the different part of the genome, construct dsRNA libraries, introduced into different individual cells, the protein expression can be changed to filter the gene and determine gene function[11-15]. Therefore, our experiments adopted the external synthesized interference RNA, specifically inhibit the eosinophil CCR3 expression, observed the EOS CR3 and eosinophil granule proteins-MBP, ECP, EPO mRNA changes in mice bone marrow, peripheral blood and nasal lavage fluid EOS.

In our previous work, we first estabalish the animals model, calculated by behavioral scoring system(>5 is a successful animal model), we found that the average score of group 2 and group 3 were all> 5. The normal group and CCR3 siRNA group only with mild scratching nose and less sneezing, score<5. HE staining showed that compared with group 1, group 2 and group 3 were with significantly mucosal epithelium, goblet cell hyperplasia and EOS and other inflammatory cells, mucous layer thickening, dilatation and congestion of the lamina propria, tissue edema, gland hyperplasia and great hypertrophy secretion. That means

the allergic rhinitis animal model can be successfully established by this method, and the application of CCR3 siRNA treatment can effectively control the occurrence of allergic rhinitis.

Our previous studies has applied lentiviral vectors to constructed simultaneously for four fragments (mCCR3-1, mCCR3-2, mCCR3-3 and mCCR3-4) CCR3 siRNA expression shuttle plasmid (pLVX-ShRNA2-mCCR3-1 +2 + 3 +4) successfully, and have a higher silencing effect which identified by QPCR on EOS CCR3 gene[16]. Detected by RT-PCR it was found that EOS CCR3 mRNA granule protein mRNA expression were high in the bone marrow, peripheral blood and nasal lavage fluid of the allergic rhinitis mice, which indicating that the EOS involved in the localization and activation of the tissue during the disease pathogenesis. In the experimental group, after treatmen of topical nasal pLVX-ShRNA2-mCCR3-1 +2 +3 +4, the test results show the corresponding EOS CCR3 mRNA and granule protein mRNA (MBP, ECP, EPO) expression was decreased, and reducing the changes of nasal cavity mucosa pathological, which indicate that the application-specific CCR3 siRNA can inhibite CCR3 expression and also decreased the eosinophil granule protein mRNA expression. This shows that the CCR3 may be involved in the whole process of the development, migration and invasion of EOS, which can not only block the CCR3 receptor expression, but also block its maturation, activation and chemotaxis, and blocking the recruitment of hematopoietic progenitor cells, mature eosinophil to sites of inflammation, improve the inflammatory response of allergic rhinitis[6,17,18].

In this study, we investigated the use of RNAi technology to treat allergic rhinitis of mice model, we found that inhibit the expression of CCR3 mRNA can reduce the eosinophil granule protein mRNA expression in the the bone marrow, peripheral blood and nasal lavage fluid, improved pathological changes in the nasal mucosa of mice, which indicated that EOS is suppressed in the development, migration and invasion , so as to relieve nasal allergy of the allergic rhinitis. Thus, based on the efficient and specific sequence interference for the target gene CCR3, we can provide some ideas for future exploration of the targeting siRNA gene therapy for allergic rhinitis.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Shi HZ. Eosinophils function as antigen-presenting cells. *J Leukoc Biol* 2004; **76**: 520-527.

[2] Wang HB, Ghiran I, Matthaei K. Airway eosinophils: Allergic inflammation recruited professional antigen-presenting cells. *Immunol* 2007; **179**: 7585-7592.

[3] Tao LH, Zhang Z, Kong WJ. Allergic rhinitis mechanism of hematopoietic regulation and eosinophils. *Int J Otolaryngol-Head & Neck Surg* 2009; **33**(2): 67-70.

[4] Denburg JA, Keith PK. Eosinophil progenitors in airway diseases. *Chest* 2008; **134**: 1037-1043.

[5] Rdinger M, Ltvall J. Eosinophil progenitors in allergy and asthma-Do they matter? *Pharmacol & Therapeut* 2009; **121**: 174-184.

[6] Fulkerson PC, Fischetti CA, McBride ML. A central regulatory role for eosinophils and the eotaxin-CCR3 axis in chronic experimental allergic airway inflammation. *PNAS* 2006; **133**(44): 16418-16423.

[7] Gong H, Li FC. Research progress on the relationship between chemotatic factors of eotaxin\CCR3 and allergic rhinitis. *J Trad Chin Med Uni Hunan* 2011; **31**(8): 75-77.

[8] Lv LL, Zhu SY, Liu PL. Expression of glucocorticoid on asthma guinea pig model eotaxin, CCR3 on regulation. *Cell & Mole Immunol J* 2009; **25**(1): 70-72.

[9] López-Fraga M, Martinez T, Jiménez A. RNA interference technologies and therapeutics: from basic research to products. *Bio Drugs* 2009; **23**(5): 305, 332.

[10]Li SF, Liu TF. Recent advances in the application of RNA interference techniques. *Chin J Exp Ani* 2009; **17**(1): 76-79.

[11]Maes T, Toumoy KG, Joos GF. Gene therapy for allergic airway diseases. *Curr Allergy Asthma Rep* 2011; **11**(2): 163-172.

[12]Meinicke H, Darcan Y, Hamelmann E. Targeting allergic airway diseases by siRNA: an option for the future. *Curr Mol Med* 2009; **9**(2): 483-494.

[13]Jiang R, Zhang G, Ma L. Construction of a stable human nasopharyngeal carcinoma cell line with down-regulated expression of β-catenin. *J Trop Med* 2011; **11**(5): 520-523.

[14]Lee CC, Chiang BL. RNA interference: new therapeutics in allergic diseases. *Curr Gene Ther* 2008; **8**(4): 236-246.

[15]Suzuki M, Zheng X, Zhang X. Novel vaccination for allergy through gene silencing of CD40 using small interfering RNA. *J Immunol* 2008; **180**(12): 8461-8469.

[16]Zhu XH, Liao B, Wang XY, Liu K, Liu YH.Construction and identification of mouse eosinophils CCR3 gene RNA interference lentiviral vector. *Chin J Otorhnolaryngol Head Neck Surg* 2013; **48**(4): 316-321.

[17]Liao B, Zhu XH, Liu YH. RNA interference in the application research on the treatment of allergic rhinitis. *J Clinical Otolaryngol Head Neck Surg* 2012; **26**(3): 141-144.

[18]Zhang L, Han DM. Attaches great importance to the research of allergic rhinitis. *Chin J Otolaryngol Head & Neck Surg* 2010; **45**(6): 441-443.