

ORIGINAL RESEARCH–ALLERGY

Decreased FoxP3 gene expression in the nasal secretions from patients with allergic rhinitis

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OBJECTIVE: To determine whether patients with allergic rhinitis have altered FoxP3 gene expression and/or mutations.

STUDY DESIGN AND METHODS: We collected nasal secretions from 14 volunteers (five of whom have allergic rhinitis) and five ENT allergy patients. Total RNA was isolated from these nasal secretions. The gene expression levels of FoxP3 were quantified by both semi-quantitative RT-PCR and real-time PCR using Actin as a housekeeping gene. The cDNA fragments amplified by RT-PCR were analyzed by DNA sequencing.

RESULTS: We found that patients with allergic rhinitis had significantly lower FoxP3 mRNA compared to nonallergic controls ($P < 0.01$). In addition, we found a point mutation in the FoxP3 gene from a patient who not only has severe allergic rhinitis, but also has asthma. This mutation locates in a highly conserved region of FoxP3 gene and partially impaired FoxP3 functions.

CONCLUSION: Our data indicate that either reduced FoxP3 gene expression or impaired FoxP3 functions are involved in the development of allergic disease in humans.

Sponsorships or competing interests that may be relevant to this article are disclosed at the end, preceding the references.

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Allergic rhinitis is a chronic inflammatory disorder characterized by an aberrant immune response to inhaled environmental antigens.¹ CD4⁺ T helper (Th) cells, which can differentiate into either Th1 or Th2 subset, play a major role in the development of allergic disorders.² Th1 cells deal directly with outside pathogens (bacteria, viruses). They secrete interleukin 2 (IL-2), IL-3, tumor necrosis factor alpha (TNF- α), and interferon gamma (IFN- γ). The Th2 cells, in contrast, modulate B-cell production of immunoglobulins and humoral immunity. They secrete IL-4, IL-5, IL-10, and IL-13. IL-4 and IL-13 induce B-cell isotype switching to IgE production, and IL-5 encourages eosinophilic inflammation, both of which are pro-allergic phenomena. Th1 and Th2 cell populations exist in a balanced feedback. In general, an abundance of the Th2 subset is associated with atopic (allergic) disease.^{3,4} About 20% of the U.S. population has the capacity to become “over-weighted” on the Th2 side.¹ The precise mechanism underlying

ing why some people respond to environmental antigens with production of Th2 cytokines, and others (nonatopic patients) do not, remains unknown.

The regulatory T cells (Treg) are a relatively newly described category of T cells (in addition to helper cells) that participate in suppressing autoreactive T cells, thus facilitating self-tolerance.⁵ Tregs constitutively express IL-2 receptor alpha chain (CD25). These CD4⁺CD25⁺ cells appear to play a major role in the suppression of both autoimmune and allergic disease.⁶ Early evidence suggests that although CD4⁺CD25⁺ cells appropriately suppress antigen induction of Th2 cells in nonatopic patients, either insufficient amount or impaired functioning of CD4⁺CD25⁺ cells in allergic patients permits antigen induction of the Th2 cells, ultimately resulting in allergic symptoms.⁷

FoxP3 is a master switch gene for many Treg cells, particularly CD4⁺CD25⁺. This transcriptional regulator determines levels and activity of CD4⁺CD25⁺ Tregs.⁸ Mice with FoxP3 deficiency have marked allergic dysregulation, with extremely high IgE levels and eosinophilia. Humans with FoxP3 mutations exhibit IPEX syndrome (immune dysregulation polyendocrinopathy enteropathy-X-linked) or XLAAD syndrome (X-linked autoimmunity allergic dysregulation). Both have excessive autoimmunity with high IgE levels, peripheral eosinophilia, Th2 skewing, and severe allergic inflammation with eczema and food allergy.^{9,10}

The goal of this study is to further delineate the role of the FoxP3 gene in allergic rhinitis in humans by exploring the correlation of FoxP3 expression with the development of this disease. We found that patients with allergic rhinitis have reduced FoxP3 gene expression. A point mutation was found in a patient with multiple severe allergic diseases including allergic rhinitis and asthma. Our results demonstrated a direct link between the impaired FoxP3 functions with allergic rhinitis in humans.

MATERIALS AND METHODS

Population and Sample Size

Approval was obtained from the Institutional Review Board at the University of Missouri and all subjects were recruited

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from among the staff and patients of the otolaryngology–head and neck surgery clinic at the University of Missouri Medical School in Columbia. Given that we have no prior information such as means and standard deviations about FoxP3 levels in nasal secretions, traditional power and sample size calculations are not possible. Specimens from 19 subjects were collected for initial analysis.

Inclusion and Exclusion Criteria

All subjects were 18 years of age or older. Smokers and patients with comorbidities requiring systemic steroids were excluded. All participants filled out a questionnaire regarding allergy history, use of nasal inhaled steroids, and positive skin or in vitro testing for allergies. If the volunteers reported a history of inhalant allergies and had a prior positive allergy skin prick test they were grouped in the allergic category. Those without a history of allergic rhinitis were placed in the nonallergic category. An additional five volunteers about to begin immunotherapy were recruited randomly from the patient population of the otolaryngology–head and neck surgery clinic at the University of Missouri Medical School to increase the allergic population size. These patients filled out the same questionnaire regarding prior skin testing, allergy history, and nasal steroid usage, in addition to undergoing prick testing with antigens most commonly encountered in the geographic region. The decision to pursue immunotherapy was made prior to recruitment into the study based on continued symptomatic complaints despite maximal medical therapy.

Collection of Nasal Secretions

Normal saline solution (3 mL) was sprayed into each nostril, while the patient's head was inclined forward 45 degrees. Passively dripped fluid was collected for 1 minute, and then the subject gently blew accumulated nasal secretions into the collection container. The process was repeated for the other nostril. Fluid from both sides was collected together. Containers with collected fluids were placed on ice and transported immediately to the laboratory for processing.

Semi-Quantification by RT-PCR

Semi-quantification of FoxP3 expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR). The cells were isolated by centrifugation of nasal secretions. Cells were washed in phosphate-buffered saline and lysed in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted and was reverse transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). cDNA was amplified in the presence of specific primers and probes for FoxP3 and β -actin. The primer sequences for FoxP3 were 5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TTCTCACAACCAGGCCACTTG-3'; for β -actin, 5'-TGAAGAGCTACTGTAATGATCAG TCAAC-3' and 5'-AGCAAGCTTGCAACCTTAACCA-3'. Each PCR was performed in triplicate, using the following conditions: 5 minutes at 95°C, followed by 35 cycles of

15 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C.

Real-Time PCR Analysis of FoxP3 Expression

Quantitative real-time RT-PCR was performed on a Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). β -actin gene was used as a reference for sample normalization. Mouse FoxP3 primers and probe and all β -actin primers and probe were purchased as forms of Assay-on-Demand (Applied Biosystems). Standard amplification protocol was used following the manufacturer's description.

Cloning Human FoxP3 Gene and Generating its Mutation

Human FoxP3 cDNA was amplified by using RT-PCR with a forward primer that was engineered to contain a Bgl II site prior to the start codon (5'-CAGATCTATGCCCAACCCCAGGCCTGGCA-3') and a reverse primer containing an EcoRI site (5'-CGAATTCAGGGGCCAGGTGTAGGGTTGGAAC-3'). The cDNA was cloned into the mammalian vector pMIG that we have modified by adding an N-terminal HA epitope. The FoxP3 point mutation was generated by PCR using a mutation kit from Stratagene (Cedar Creek, TX). All the plasmids' DNA were confirmed by DNA sequencing.

Dual Luciferase Assay

HEK 293 cells in 12-well plates were transfected with pRL-TK (Promega, Madison, WI) and pIL-2 luciferase, along with various expression plasmids, using the Lipofectamine transfection reagent (Invitrogen Life Technologies, San Diego, CA). The pRL-TK plasmid contains the *Renilla reniformis* (sea pansy) luciferase gene under the transcriptional control of the herpesvirus thymidine kinase promoter and constitutively expresses low levels of renillar luciferase. Transfected cells were lysed, and the luciferase activities in the cell lysates were analyzed using a Dual Luciferase Reporter assay kit (Promega, Madison, WI). Luciferase activity was measured as relative light units (RLUs) using a luminometer (Turner BioSystems, Inc., Sunnyvale, CA).

Data Analysis

Statistical analysis was performed using the statistical software SPSS (Chicago, IL). The Mann-Whitney *U* test was used, with *P* values <0.05 considered significant.

RESULTS

The Expression Levels of FoxP3 are Decreased in the Nasal Secretions from Patients with Allergic Rhinitis

To investigate whether the FoxP3 expression levels are altered in the patients with allergic rhinitis, we collected nasal secretions from 14 volunteers, five of whom have allergic rhinitis. Total RNA was isolated from these nasal secretions and dissolved in 20 μ L water. The concentra-

tions of the total RNA were very low due to limited numbers of cells that could be obtained from the nasal secretions. We thus used each of 5 μ L of total RNA for reverse transcriptions using oligo-dT as primer. Both FoxP3 and actin cDNA were detected from all the 14 samples (Fig 1A). The densities of these PCR bands were quantified using a PhosphoImage program. The average mRNA levels of actin were higher in samples from these donors with allergic rhinitis, suggesting more secreted cells were obtained from those donors (Fig 1A and data not shown). The ratios of FoxP3 mRNA levels with actin mRNA levels are significantly reduced in the nasal secretions from donors with allergic rhinitis (Fig 1B,C). We further confirmed our results using a real-time PCR approach and analyzed those 14 samples as shown in Figure 1 and five samples collected from our ENT patients who have allergic rhinitis. Figure 2 shows FoxP3 mRNA copy numbers in the samples that contain 1000 copies of actin mRNA. Similar to our RT-PCR results, the average mRNA levels in the secretion of donors with allergic rhinitis were dramatically reduced (Fig 2). Our results demonstrating a reduced FoxP3 expression in those patients with allergic rhinitis indicate that decreased Treg cells in the nasal secretions is possibly correlated to the development of allergic rhinitis.

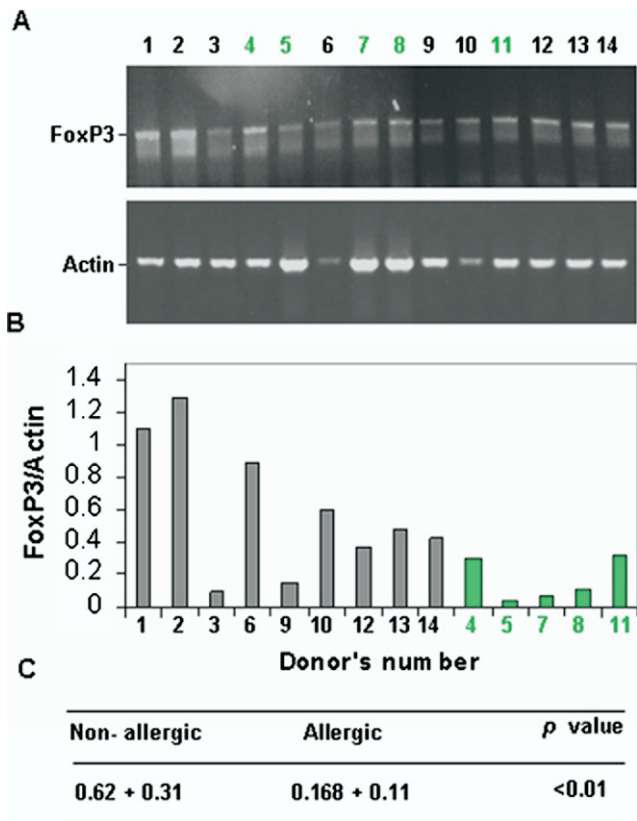


Figure 1 Expression levels of FoxP3 in the nasal washing from nonallergic and allergic subjects. The average ratios from nonallergic and allergic subjects were calculated and statistically analyzed by the Mann-Whitney *U* test.

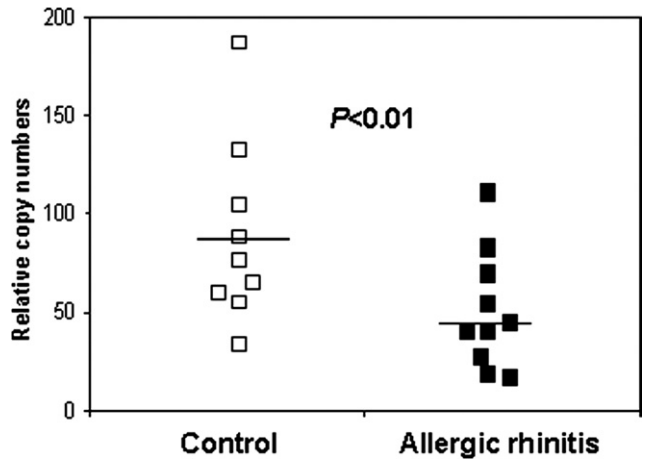


Figure 2 Determination of FoxP3 expression in the nasal secretions by real-time RT-PCR. Data were statistically analyzed by the Mann-Whitney *U* test.

Identification of FoxP3 Mutation from a Patient with Severe Allergic Rhinitis and Asthma

Mutations of FoxP3 gene have been found in patients with autoimmune disease.¹¹ To determine whether any of the patients with allergic rhinitis have mutations in FoxP3 mRNA, we amplified FoxP3 cDNA and sequenced the products. A point mutation of FoxP3 was found in the nasal secretion from one out of 10 donors who have allergic rhinitis (Fig 3A). No mutations in the FoxP3 gene were found in any of the healthy controls. This was a G-to-T mutation, which subsequently caused an arginine-to-tryptophan (R-to-W) mutation at 146 amino acid position of FoxP3 open reading frame. Unlike most of the point mutations found in the C-terminus, particularly the forkhead DNA binding domain, of FoxP3, this mutation locates in a highly conserved region in the transcriptional activation (TA) domain of FoxP3 (Fig 3B,C). Interestingly, the patient who carries this mutation has not only allergic rhinitis but also severe asthma, suggesting a possible link of this mutation with allergic disease development.

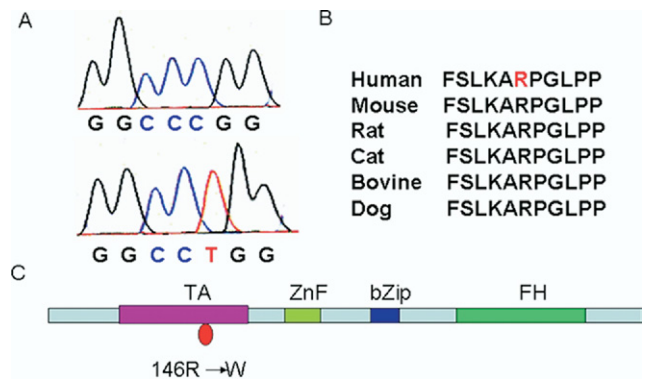


Figure 3 The R146W FoxP3 point mutation from a patient with severe rhinitis and asthma.

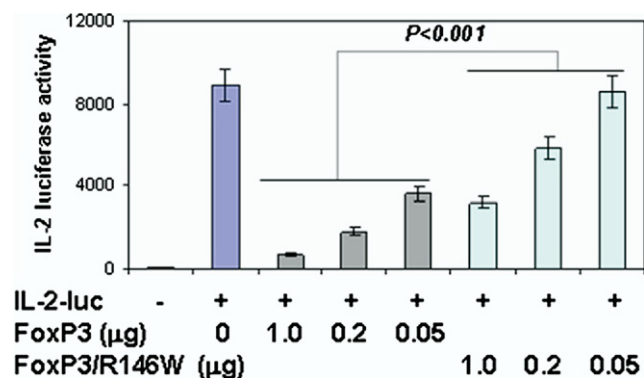


Figure 4 The R146W mutation impairs the suppressive functions of FoxP3. Error bars represent data from five independent experiments (means + SD).

R146W Mutation Partially Impairs FoxP3 Functions

We next determined whether mutation of FoxP3 at 146 arginine affects its function. Human FoxP3 gene was cloned into an expression vector and its R146W mutant was generated. Expression of wild-type FoxP3 dramatically inhibited IL-2 luciferase activities as previously reported.¹² The inhibitory activity of FoxP3 mutant is significantly impaired ($P < 0.001$) (Fig 4). These results indicate that this point mutation impairs FoxP3 functions in T cells, suggesting that the Tregs carrying this FoxP3 mutant may not be able to sufficiently suppress Th2 responses in the patient.

DISCUSSION

In this study we have demonstrated a statistically significant decrease in the expression of FoxP3 mRNA in the nasal secretions from allergic as compared to nonallergic subjects. Since FoxP3 is a specific lineage molecule of regulatory T cells, such a decrease would result in decreased regulatory T cells. In addition, we identified a point mutation in the FoxP3 gene from a patient with severe allergic diseases including rhinitis and asthma. This mutation locates in a highly conserved region of FoxP3, which could potentially impair its function. Indeed, the inhibitory activity of FoxP3/R146W mutant in suppression of IL-2 luciferase activity is partially impaired. Thus, reduced FoxP3 gene expression or impaired FoxP3 functions are potentially responsible for the development of allergic diseases in humans.

Tregs play a central role in suppressing autoimmunity and allergic diseases; thus, impaired Treg functions causes immune diseases.¹¹ The reduced expression of FoxP3 gene in the nasal secretions from patients with allergic rhinitis can be caused by two mechanisms, namely, either impaired Treg development or failed recruitment of Treg to nasal mucosal membrane. Indeed, recent studies have demonstrated the reduced CD4⁺CD25⁺ regulatory T cell populations in blood from patients with allergic asthma, suggesting

that impaired Treg development, which causes reduced Treg numbers in the circulated blood, is involved in the disease.¹³ The existence of FoxP3 gene in the nasal secretions provided a direct evidence for the distributions of Tregs into the nasal mucosal membranes. This distribution could play a critical role in suppressing Th2 responses and preventing allergic response. Sather et al recently found that altering Treg cell distribution in vivo leads to the development of tissue-specific inflammatory disease, and the expression of T-cell homing factors, such as CCR4, CCR7, and CD62L, contribute to the inflammatory lung disease development.¹⁴ Given the fact that the altered T-cell homing factors can cause allergic rhinitis in humans,¹⁵⁻¹⁸ it will be interesting to further investigate whether the reduced FoxP3 gene expression in the nasal mucosal membrane of the allergic rhinitis patients is due to the altered Treg distributions.

As a lineage-specific factor of Tregs, FoxP3 expression is sufficient for Treg development and functions.¹⁷ Deletion mutations in FoxP3 gene, which produce complete nonfunctional FoxP3 proteins, often cause severe autoimmunity both in mice and in humans and result in the early death of these carriers.¹⁸ Point mutations of FoxP3 that cause partial loss of FoxP3 function have been found in patients with autoimmune disease.¹¹ The major symptoms of patients with FoxP3 mutations are autoimmune, such as insulin-dependent diabetes mellitus (IDDM) and thyroiditis, which develop in association with autoantibodies. However, some of these patients have high IgE levels in their sera.¹⁰ Most of mutations locate in the C-terminus of FoxP3, particularly its DNA-binding domain.¹⁶ The R146W locates in a highly conserved region of the N-terminus of FoxP3. Notably, the patient who carries this mutant has severe asthma. This mutation may cause the allergic diseases of the patient. Our laboratory is currently investigating how this mutation alters FoxP3 functions. In addition, about 30% of patients with allergic rhinitis have asthma;¹⁹ it will be thus interesting to determine whether a higher frequency of FoxP3 mutation exists in those patients.

Immunotherapy has been widely used in clinics for the treatment of allergic rhinitis by repeatedly immunizing patients with allergens. Most of the patients who receive immunotherapy have reduced allergic symptoms. The mechanisms underlying these effects are not fully understood. The Th2 tolerance induced by repeated allergen challenging has been considered as one of the mechanisms. Recent studies have suggested that repeated stimulation of CD4⁺ T cells can also induce Tregs.²⁰ The increased IL-10 and TGF- β levels in the sera from the patients who received immunotherapy have also been observed.²¹ TGF- β is an initiator for Treg differentiation, and IL-10 is a cytokine produced by Tregs to suppress autoimmunity.²² However, the direct evidence that immune therapy effects Treg differentiation is still missing. Future research will delineate whether and how the expression of FoxP3 genes changes during immunotherapy.

Allergic rhinitis affects up to 20% of the U.S. population and comprises a significant portion of health care visits and costs. One of the mainstays of treatment is immunotherapy. A clear understanding of the genetic mechanisms of this disease remains unknown. FoxP3 is a master switch gene for CD4⁺CD25⁺ T regulatory cells. CD4⁺CD25⁺ insufficiency or dysfunction is associated with atopy via more robust Th2 cell activation by antigen exposure. The T cell subset called Treg cells can suppress both antigen-specific and nonspecific immune allergic (Th2) responses. In this study, we were able to demonstrate for the first time that human allergic patients have decreased FoxP3 levels in nasal secretions compared to nonallergic patients. This would result in markedly decreased CD4⁺CD25⁺ cell numbers, which has been shown to be associated with allergic disease. Future research will elucidate the role of FoxP3 and Tregs during the course of immunotherapy for the treatment of allergic rhinitis.

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AUTHOR CONTRIBUTIONS

Sang-Myeong Lee, performed some experiments; **Bei-Xue Gao**, performed some experiments; **Matthew Dahl**, sample collections and preparation of the manuscript; **Karen Calhoun**, study design; **Deyu Fang**, study design and drafting of the manuscript.

FINANCIAL DISCLOSURE

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