


รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาการกลายพันธุ์ของยีน STAT6 ต่อการเกิดโรคภูมิแพ้

โดย ผู้ช่วยศาสตราจารย์แพทย์หญิง นริศรา สุรทานต์นนท์

เมษายน 2562

สัญญาเลขที่ MRG60800172

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สังกัดหน่วยภูมิแพ้และภูมิคุ้มกัน ภาควิชากุมารเวชศาสตร์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Abstract (บทคัดย่อ)

Project Code : MRG60800172

Project Title : การศึกษาการกลายพันธุ์ของยีน STAT6 ต่อการเกิดโรคภูมิแพ้

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Objective : To investigate the functional consequences of the STAT6 mutation

Methods : We described a child who had idiopathic anaphylaxis in infancy, atopic dermatitis and allergic eosinophilic gastroenteritis with protein-losing enteropathy. His father had atopic dermatitis and food allergy. Both were heterozygous mutations in *STAT6* DNA binding domain. Flow cytometric analysis, cytokine measurements by ELISA/ Luminex assays from the patient samples, luciferase assay and immunofluorescence in *STAT6* mutants compared to wide type transfectants were performed to prove that the mutation resulting in activation of STAT6.

Results : Various experiments confirmed that the mutants are pathogenic. 3-D Structural modelling of STAT6 revealed that the *STAT6* mutation is located at the surface of the STAT6 DNA binding region, potentially having a stronger binding affinity. Immunofluorescence study confirmed that mutant *STAT6* preferentially localized in the nucleus. Constitutively DNA binding activation of STAT6 mutants was also detected through HEK293T cell luciferase assays. Flow cytometric-based analysis of T helper cell populations and intracellular cytokine measurements from patient cells showed that immune responses of the patient directed toward a

Type 2 T-cell phenotype. T helper cell type 2 and IL-4+ T cell populations were increased while lack of IFN-gamma production (T helper cell type 1 phenotype) was found in the patient. Gastric organoids of the patients secreted eotaxin- 2 spontaneously while further enhanced response was found after IL-4 treatment.

Summary : Our study demonstrated, for the first time, the gain-of-function *STAT6* mutation as a new human disease gene for an early onset allergic disease which may lead to a better understanding of the pathophysiology of allergic diseases and therapeutic intervention in the future.

Comments for the future research : Future study should be performed to explore if the *STAT6* mutation could be a genetic susceptibility for allergic diseases by sequencing the *STAT6* in a cohort of subjects who have early onset multiple episodes of anaphylaxis and also to explore the potential treatment for the patient with *STAT6* mutation for examples an anti-STAT6 agent or gene therapy.

วัตถุประสงค์ เพื่อศึกษาผลของการกลายพันธุ์ของยีน STAT6 ต่อการแสดงออกของโรคภูมิแพ้

วิธีทดลอง กลุ่มวิจัยของเราได้ตรวจพบครอบครัวซึ่งประกอบด้วย คนไข้ชายอายุ 2 ปีซึ่งมีอาการแพ้อย่างรุนแรงแบบไม่มีสาเหตุกระตุ้นชัดเจน (idiopathic anaphylaxis) มีผื่นภูมิแพ้ผิวหนัง และมีอาการแสดงของการแพ้ในระบบทางเดินอาหารในกลุ่มอาการที่เรียกว่า allergic eosinophilic gastroenteritis ทำให้มีภาวะโปรตีนรั่วในลำไส้ บิดาของเด็กมีผื่นภูมิแพ้ผิวหนังและอาการแพ้อาหารเช่นเดียวกัน กลุ่มวิจัยตรวจพบว่า คนไข้ทั้งสองคนมีการกลายพันธุ์ของยีน STAT6 ซึ่งไม่เคยมีรายงานมาก่อน กลุ่มวิจัยจึงได้ทำการตรวจวินิจฉัยด้วยวิธี Flow cytometry ตรวจวัดไซโตไคน์ด้วยวิธี ELISA และ Luminex ในคนไข้ ทดสอบด้วยวิธี immunofluorescence เพื่อดู ตำแหน่งของ STAT6 ที่มีการกลายพันธุ์และไม่มีการกลายพันธุ์ และทำการศึกษาวิจัยด้วย luciferase assay เพื่อตรวจดูคุณสมบัติการจับกับ DNA ของ STAT6 ที่มีการกลายพันธุ์ ทั้งนี้เพื่อพิสูจน์ว่า การกลายพันธุ์ของยีน STAT6 ได้ส่งผลให้คนไข้มีอาการแสดงของโรคภูมิแพ้

ผลการทดลอง เมื่อพิจารณาตำแหน่งของการกลายพันธุ์ของ STAT6 ของคนไข้ใน 3-D Structural modelling พบว่า การกลายพันธุ์อยู่ในตำแหน่งที่ใกล้กับตำแหน่งที่จับกับนิวเคลียส ทำให้เชื่อว่าน่าจะส่งผลให้ STAT6 จับกับ DNA ได้แน่นมากขึ้น ผลการย้อม Immunofluorescence พบว่า โมเลกุล STAT6 ที่มีการกลายพันธุ์มักจะพบอยู่ในนิวเคลียสมากกว่า STAT6 ที่ไม่มีการกลายพันธุ์ รวมทั้งมีการเพิ่มขึ้นของ DNA binding activation เมื่อทำการตรวจด้วย luciferase assays ใน HEK cell line นอกจากนี้ ผลของ Flow cytometric-based analysis พบว่า คนไข้มีปริมาณ Type 2 T-cell มากกว่าคนปกติและคนที่เป็นโรคภูมิแพ้แต่ไม่มีการกลายพันธุ์ของยีน STAT6 นอกจากนี้ T cell ของคนไข้ยังหลั่ง IL-4 ในปริมาณที่มากกว่าคนปกติ แต่ไม่สามารถสร้าง IFN gamma ได้ ซึ่งเป็นสิ่งสนับสนุนว่า ภูมิคุ้มกันของคนไข้เบี่ยงเบนไปในทางการตอบสนองแบบภูมิคุ้มกันทาง T helper 2 นอกจากนี้ organoid ที่สร้างจากเซลล์กระเพาะของคนไข้สามารถสร้าง eotaxin-2 ได้เองโดยไม่ต้องถูกกระตุ้นด้วย IL-4 แต่เมื่อถูกกระตุ้นด้วย IL-4 ปริมาณ eotain-2 ยิ่งเพิ่มมากขึ้น และมีปริมาณที่มากกว่า organoids ของคนปกติ

สรุปและวิจารณ์ผลการทดลอง จากผลการทดลองสามารถยืนยันว่า STAT6 ซึ่งมีการกลายพันธุ์ในคนไข้ส่งผลให้มีการทำงานของโมเลกุล STAT6 เพิ่มขึ้น และเป็นสาเหตุที่ทำให้เกิดอาการแสดงของโรคภูมิแพ้ในคนไข้ ก่อให้เกิดองค์ความรู้ใหม่ว่า การกลายพันธุ์ของยีน STAT6 สามารถทำให้เกิดอาการแสดงในคนไข้ภูมิแพ้ได้ นำไปสู่การพัฒนาความรู้ความเข้าใจในการเกิดโรคภูมิแพ้และแนวทางการรักษาใหม่ในอนาคต

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต ได้แก่ การศึกษาอุบัติการณ์ การกลายพันธุ์ของยีน STAT6 ในกลุ่มประชากรโรคภูมิแพ้ และการทดลองหาแนวทางการรักษาคนไข้ที่มีการกลายพันธุ์ของยีนSTAT6 ไม่ว่าจะเป็นการให้ยาที่มีผลลดการทำงานของ STAT6 รวมทั้งการรักษาด้วย gene therapy

Keywords : STAT6, idiopathic anaphylaxis, gain-of-function mutation, early onset allergy

Figure 1. Clinical and immunologic phenotypes of the index case.

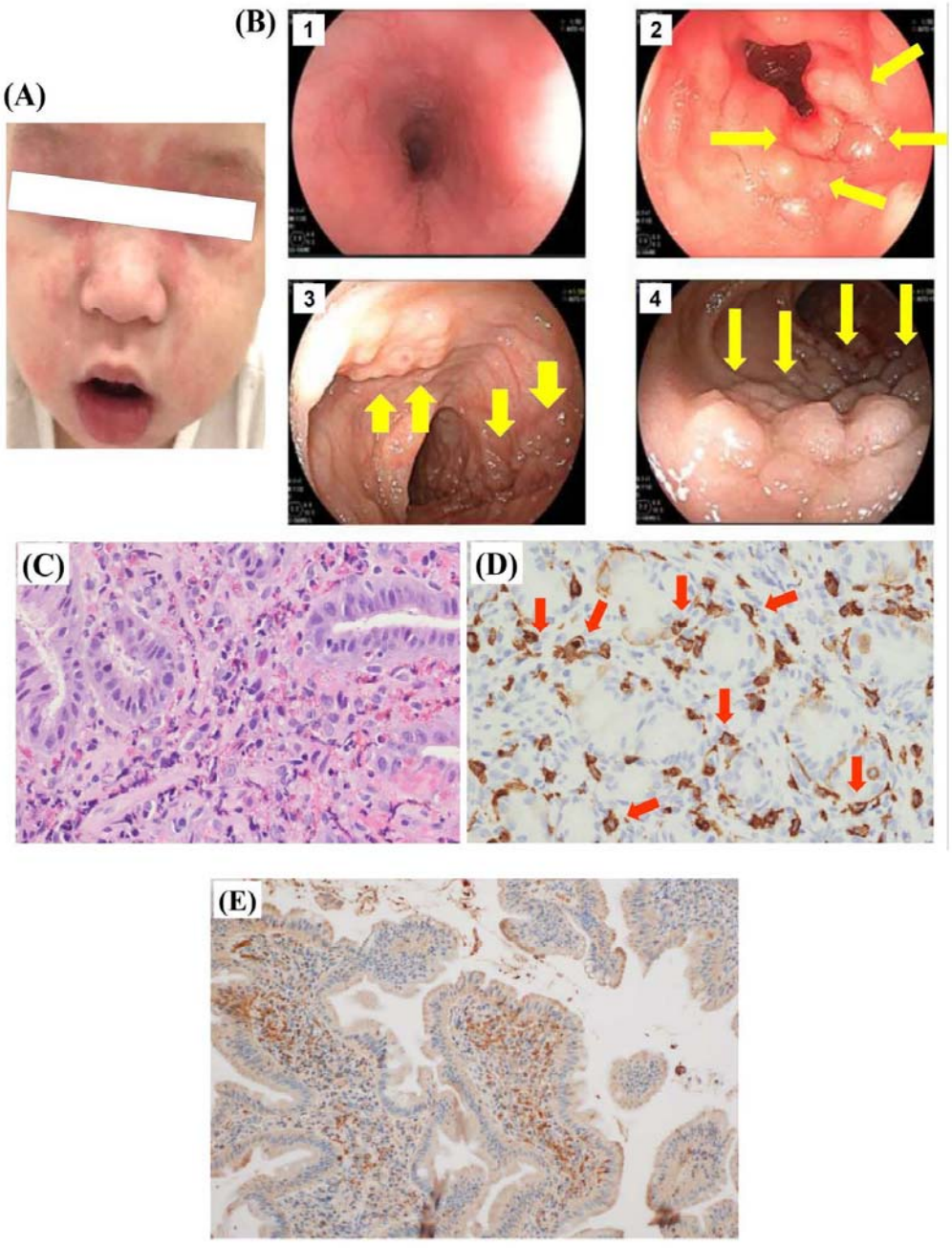


Figure 2. Heterozygous mutation of *STAT6* in the DNA binding domain.

Figure 2A

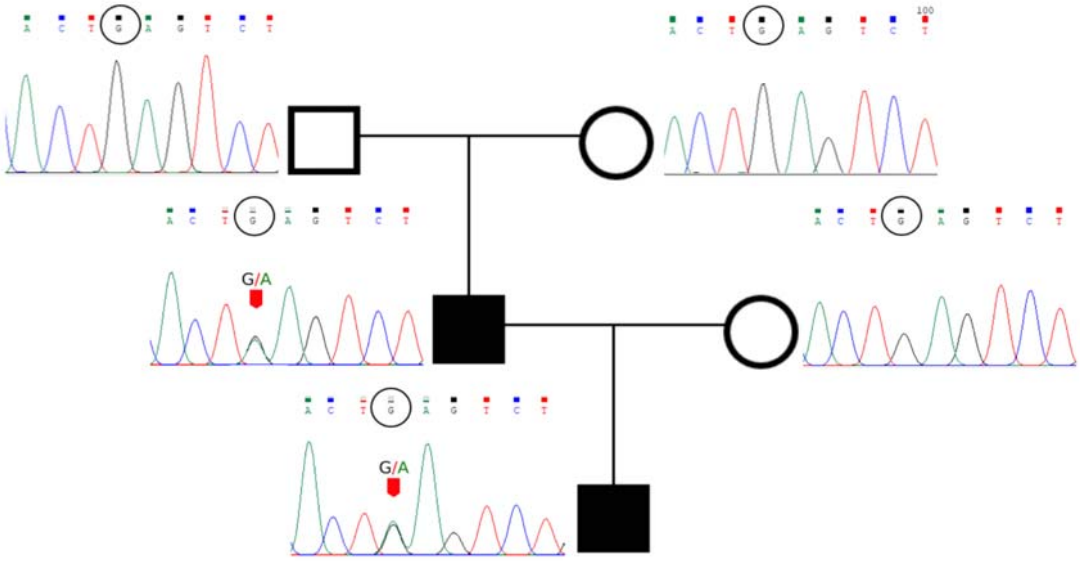


Figure 2B

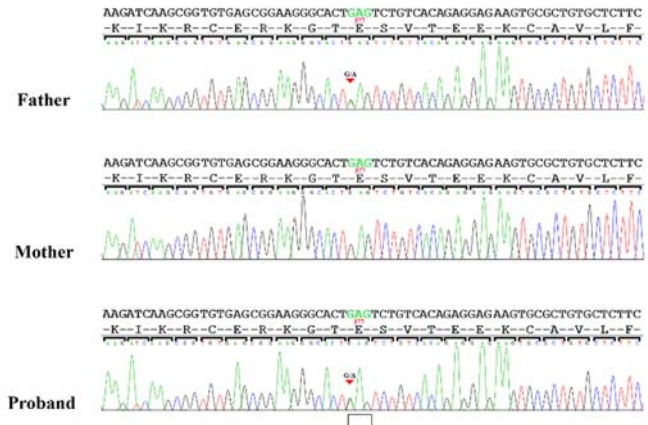


Figure 2C

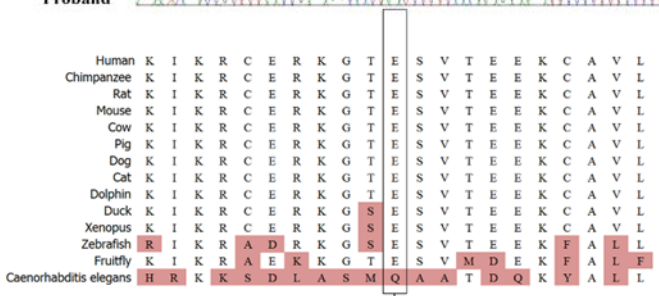


Figure 2D

Figure 2E

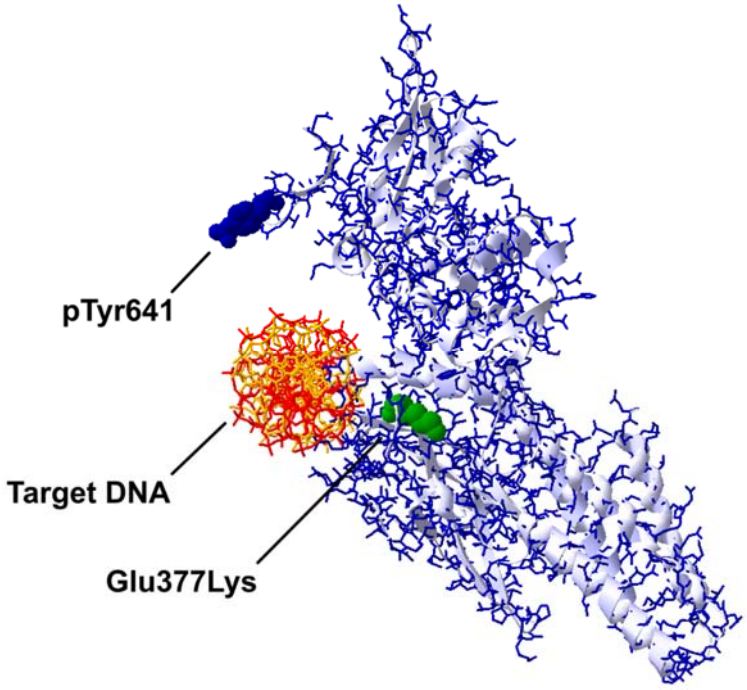


Figure 2F

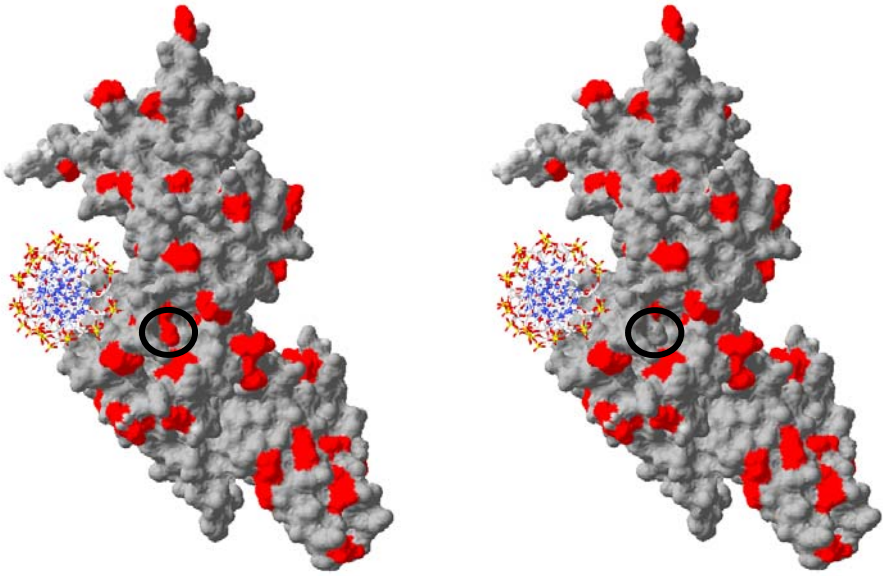


Figure 2G

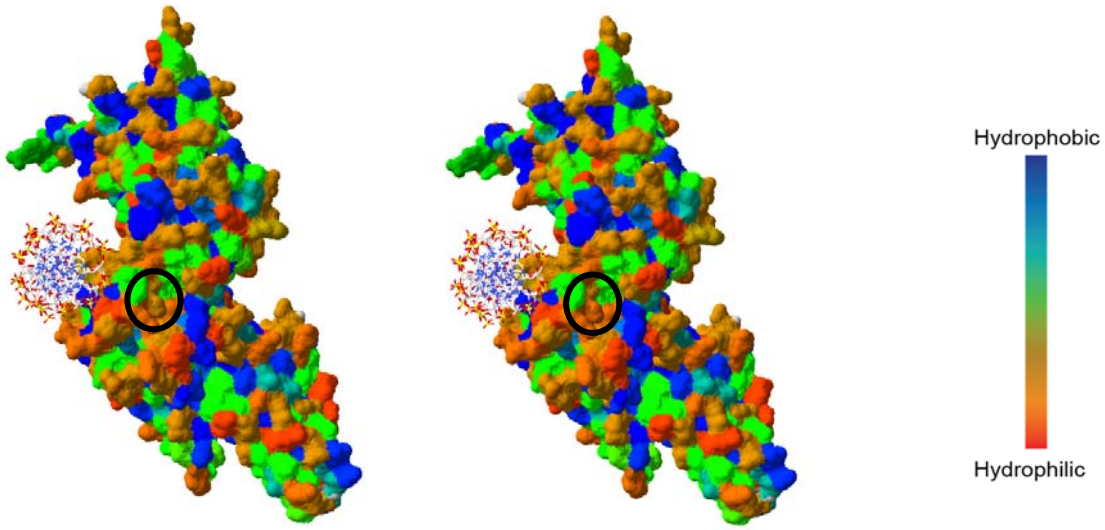


Figure 3. Mislocalization of STAT6 mutant

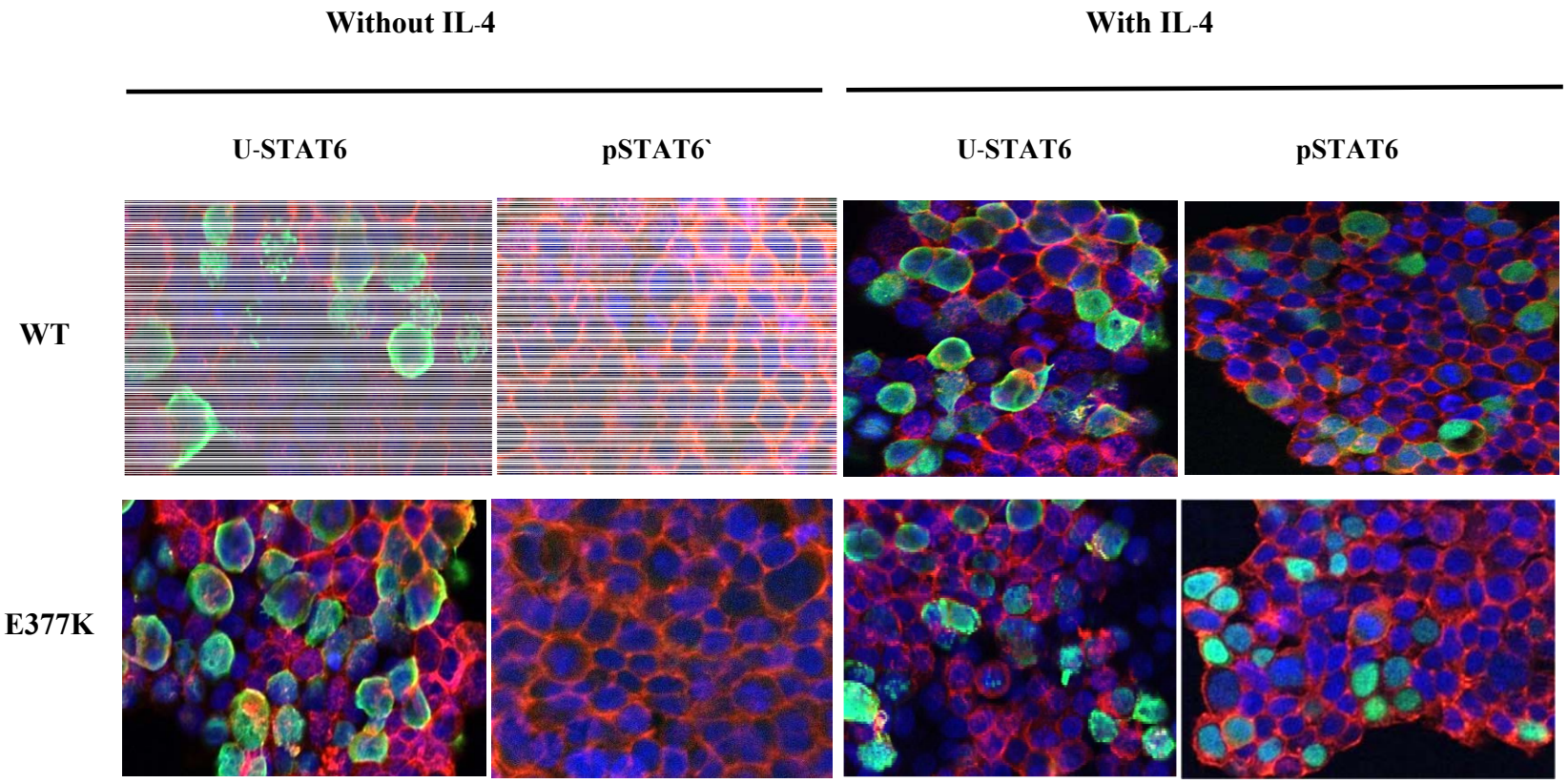


Figure 4. STAT6 mutation is intrinsically activating and partially STAT6 independent

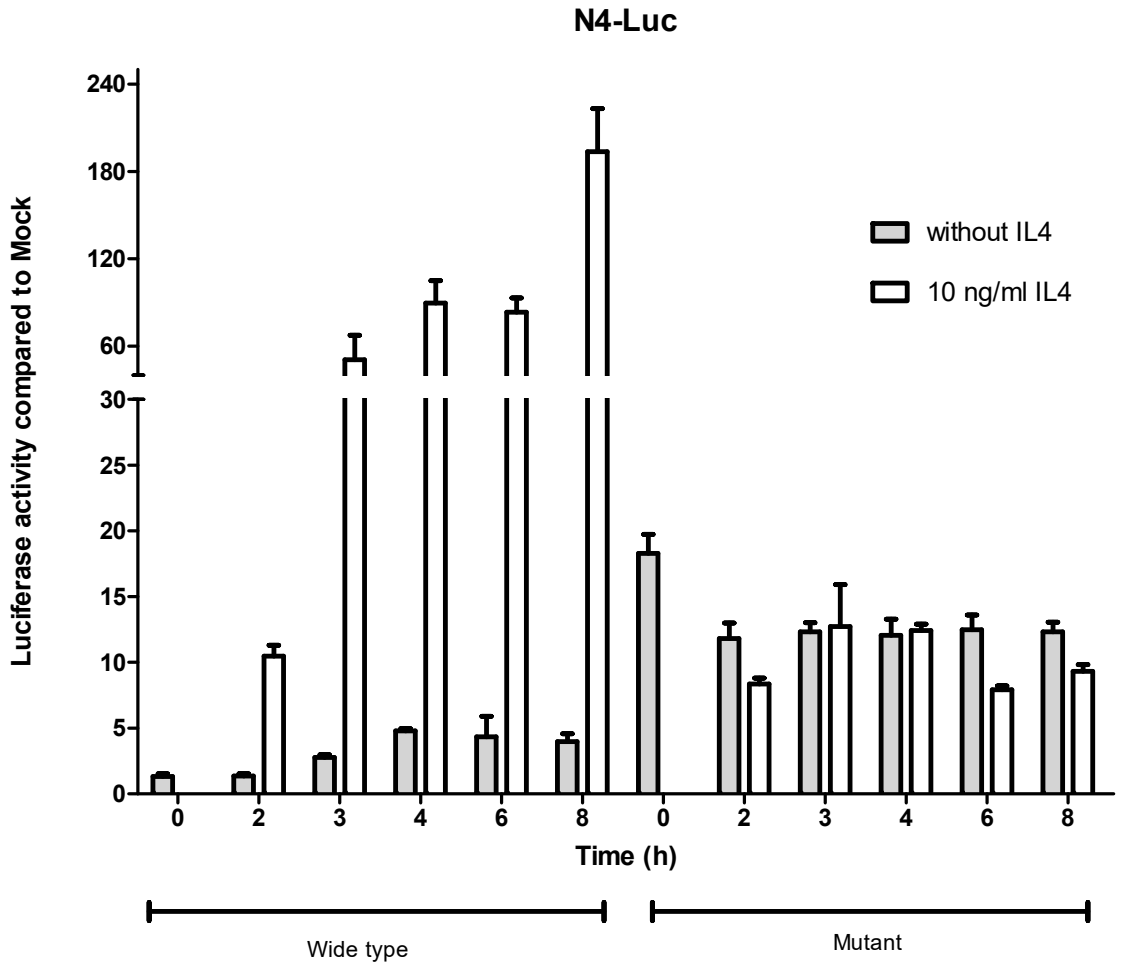


Figure 5. Gastric organoids of the index case showed enhanced allergic responses.

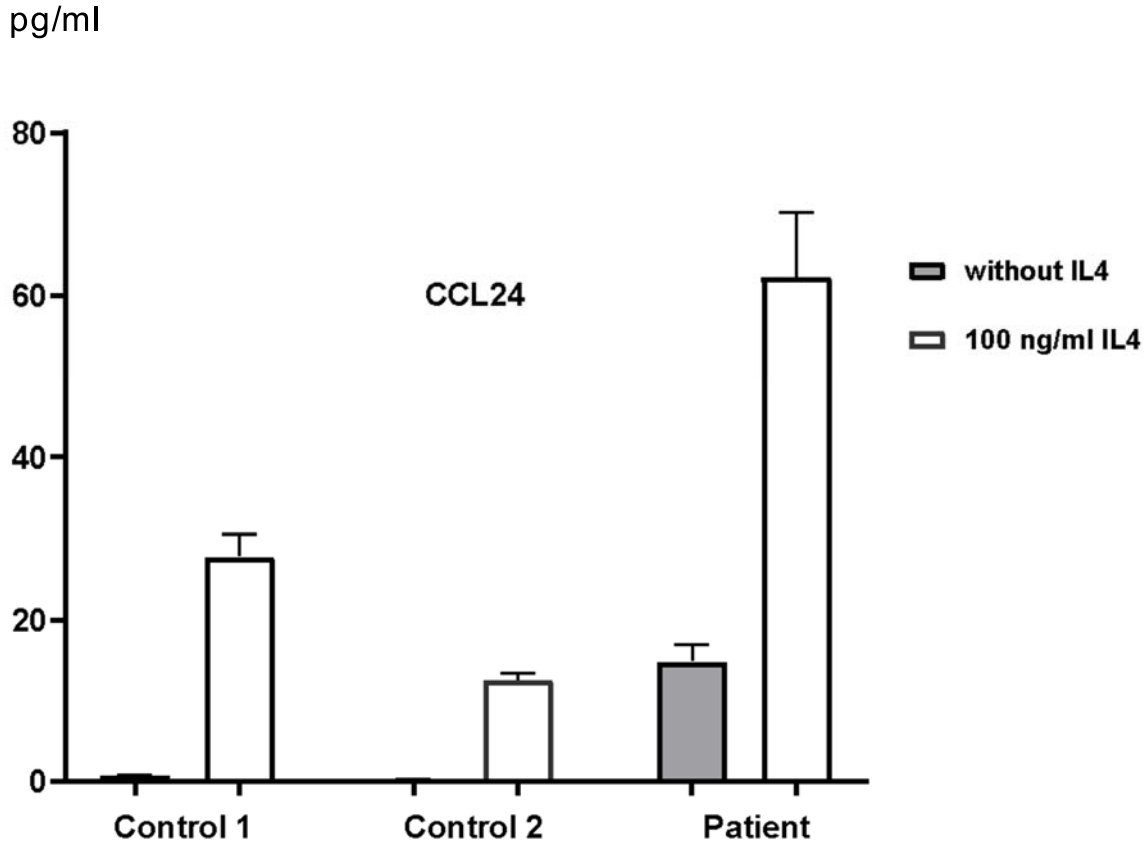
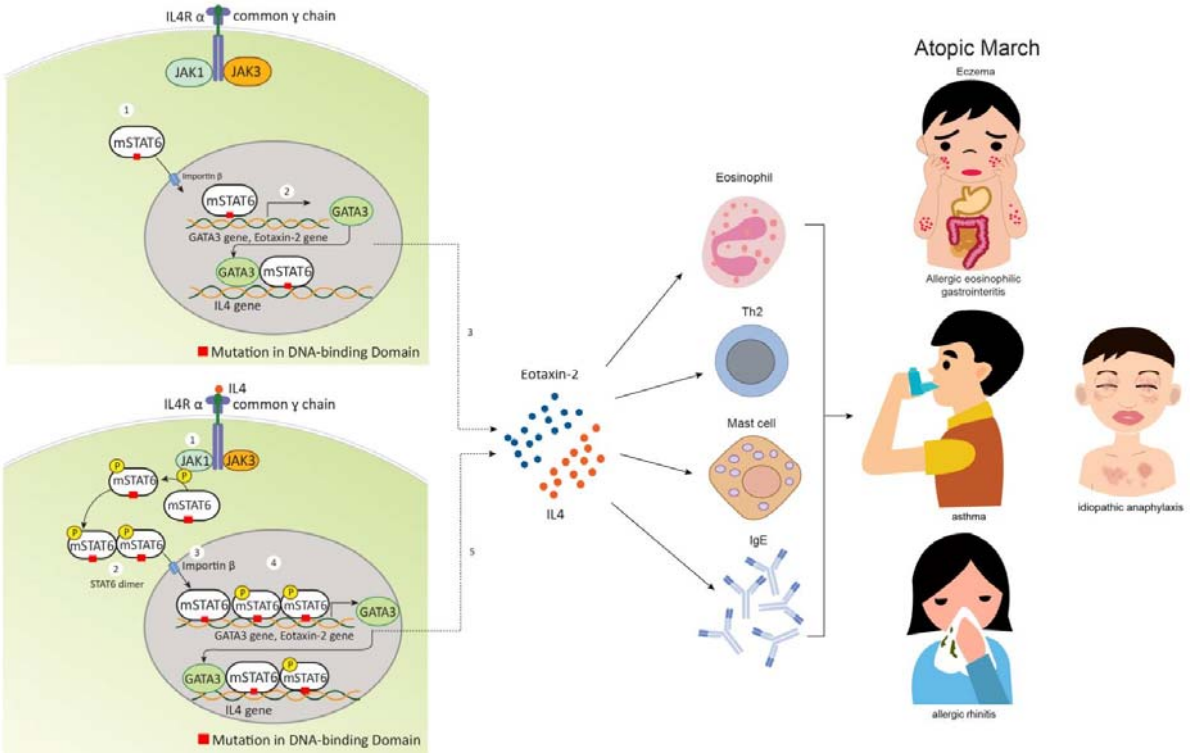


Figure 6. Illustrated mechanisms of STAT6 mutant links to the clinical phenotypes



Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ:

   manuscript in preparation ดังไฟล์แนบ

2. การนำผลงานวิจัยไปใช้ประโยชน์:

   งานวิจัยนี้มีศักยภาพที่จะได้รับการตีพิมพ์ในวารสารระดับนานาชาติ เนื่องจากเป็นองค์ความรู้ใหม่ มีคุณประโยชน์ด้านวิชาการ นำไปสู่การพัฒนาความรู้ความเข้าใจในการเกิดโรคภูมิแพ้และแนวทางการรักษาใหม่ในอนาคต

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร):

   จะมีการเผยแพร่ผลงานโดยการเสนอผลงานในที่ประชุมวิชาการระดับชาติและระดับนานาชาติหลังจากที่มีการตีพิมพ์ผลงานแล้ว

A *STAT6* Gain-of-Function Mutation Is Associated with a Familial Early Onset Allergic Disease

Summary:

Mutations in STAT6 have never been found to cause a monogenic disorder in humans. We described a child who had idiopathic anaphylaxis in infancy, atopic dermatitis and allergic eosinophilic gastroenteritis with protein-losing enteropathy. His father had atopic dermatitis and food allergy. Both were heterozygous mutations in STAT6 DNA binding domain. Various experiments suggested that the variant is a gain-of-function mutation. Our study demonstrated, for the first time, the gain-of-function STAT6 mutation as a new human disease gene for an early onset allergic disease which may lead to a better understanding of the pathophysiology of allergic diseases and therapeutic intervention in the future.

Introduction:

Allergic diseases are complex conditions resulting from the interaction of genetic and environmental factors. Several genes display known biological plausibility and functional consequences potentially implied to the disease development [1]. Interleukin (IL)-4/IL-13 pathway is one of the key players in allergic inflammation. It plays crucial roles in the development of type 2 T helper cells, IgE+ producing B cells, eosinophilic infiltration and mast cell activation. Signal transducer and activator of transcription 6 (STAT6) is a primary transcription factor acting downstream of IL-4/IL-13 [2]. In physiologic conditions, STAT6-dependent signalling is tightly regulated. Following IL-4 or IL-13 binding to cell-surface receptors, STAT6 molecule is phosphorylated, dimerised and translocated into the nucleus. STAT6 dimer then binds to the DNA motifs and regulates transcription of specific target genes. Previous studies showed that hyperactivity and single nucleotide polymorphisms of STAT6 related to the susceptibilities of asthma, atopic dermatitis, food-induced anaphylaxis, autoimmune diseases and cancers [3, 4]. Somatic mutations in STAT6 has been described in cancers such as follicular lymphoma [5], primary mediastinal B-cell lymphoma [6] and non-small cell lung cancer [7], but not an allergy. Mutations in *STAT6* have never been reported as a monogenic disorder in humans. Here we described a family with a *STAT6* mutation manifested with severe allergic manifestations. The genetic and functional data provide evidence that constitutive activation of *STAT6* leads to a severe allergic phenotype.

Results:

Phenotypes of the patients

Our index case is a 14-month-old Thai boy, presented with idiopathic anaphylaxis in infancy, atopic eczema, enamel hypoplasia and allergic eosinophilic gastroenteritis with protein-losing enteropathy (PLE). He had dry skin and extensive eczematous lesions since two months old. Otherwise, he was well until the age of 7 months when six episodes of diffuse erythema, facial and lips swelling, and difficulty of breathing from unidentified triggers started. All episodes required hospitalization. Erythema and facial swellings occurred in one episode of anaphylaxis were shown in Figure 1A. He responded well to the injection of epinephrine, chlorpheniramine, hydrocortisone and nebulized albuterol. Chronic watery diarrhoea was then started at one year of age without complaints about recurrent abdominal pain and vomiting. Physical examination at 14 months revealed normal body weight and height and mild pale conjunctivae. Oro-dental examinations exhibited severe enamel hypoplasia, dental caries, angular cheilitis, aphthous ulcers, multiple vesicles on his tongue and oropharynx, and nodules on the buccal mucosa (Supplement figure 1). No significant puffy eyelids or pitting edema was identified. However, dry, excoriation and erythematous maculopapular rash at face, scalp and trunk were noted.

Extensive investigations revealed evidence of an atopic phenotype, iron deficiency anaemia, hypoalbuminemia, hypogammaglobulinemia and allergic eosinophilic gastroenteritis with protein-losing enteropathy (PLE). Peripheral eosinophilia was present with eosinophils counts of 2,460 cells/mm3 (0-450). Hematocrit level was 28.9 % (39-51). Platelet counts were 1,487,000 cells/mm3 (150,000-450,000). A reticulocyte count was 5.0 % (1-2). Low serum iron and ferritin levels supported the diagnosis of iron deficiency; serum iron = 36 mcg/dL (60-170) and serum ferritin = 11 ng/ml (30-400). Total iron-binding capacity (TIBC) was normal; 12 (>16).

Both serum albumin and globulin were low; albumin = 2.0 g/dl (3.5-5.0), globulin = 1.4 g/dl (2.0-3.3). Serum immunoglobulin (Ig) E was marked increased; 6,370 IU/ml (normal range 20-436) while IgG and IgM levels were reduced; IgG 3.86 g/L (normal range 5.5-9.7) and IgM 0.31 g/L (0.35-0.81). Serum IgA was within the reference range for age; 0.33 g/L (0.26-0.74). Stool exam revealed neither cells nor parasites, but occult blood was positive. Stool alpha-1 antitrypsin was increased up to 32.8 mg/dL (0.25-5.22), supporting the evidence of protein-losing enteropathy. Endoscopic findings demonstrated multiple papules with linear furrow at mid to distal esophagus (Figure 1B1). Generalized swelling and erythema of gastric and duodenal mucosa with multiple polypoid-like lesions were reported (Figure 1B2). Numerous lymphonodular hyperplasia at transverse colon and terminal ileum were noted (Figure 1B3 and 1B4). Immunohistochemistry from esophageal, gastric, duodenal, ileal and colonic biopsies (Figure 1C) revealed marked eosinophilic infiltration in the lamina propria. Eosinophils infiltrated into the epithelium with reactive epithelial change were noted. Degranulation of eosinophils was diffusely seen. CD 117 immunohistochemistry (Figure 1D) highlighted numerous mast cells infiltrating in esophageal, gastric, duodenal, ileal and colonic mucosa. Vascular endothelial growth factor (VEGF), which is involved in lymphangiogenesis and relate to primary intestinal lymphangiectasia also increased in small bowel biopsies, supporting the occurring of protein-losing enteropathy (Figure 1E).

Serum tryptase of the patient was measured in one of the anaphylactic episodes. A ratio between peak tryptase and baseline tryptase supported the diagnosis of anaphylaxis; 4.57/2.95 ug/L = 1.55 (≥1.5) [8]. Levels of baseline serum tryptase did not reach the criteria of systemic mastocytosis; 2.95 ug/L (> 20). Specific IgE to food allergens was as followed; milk 28.7, wheat 90.7, soy 55.6, peanut 39.2, shrimp 71, fish 35.3 kUA/L (< 0.35). All specific IgE did not correlate with the symptoms of anaphylaxis.

Up to his last follow up at age four years, last anaphylactic episode occurred when he was 2.8-years-old. Right hydrocele was diagnosed which hydrocelectomy was done at three years old. His hypoplastic teeth were restored and fissures were sealed with glass ionomer (GC Fuji IX GP, Japan) which allergic reaction to glass ionomer has not been observed. He started to develop asthma and allergic rhinitis at the age of three. Skin prick test to aeroallergens was strongly positive to house dust mite (both *Dermatophagoides pteronyssinus, Dermatophagoides farinae*) and cat hair. He was fed by amino acid formula from 7 months to 2 years of age. His current medications were mometasone furoate nasal spray, fluticasone propionate metered-dose inhaler (MDI), salbutamol MDI, desloratadine syrup and mometasone furoate cream.

The patient is the first child of non-consanguineous parents. His mother and younger sister are healthy. The father had coarse facies, hypotrichosis, dry skin and eczema. Angioedema after eating shrimp and cashew nut was reported from the father's history. The father and other family members had normal teeth and oral mucosa. Chronic renal failure of unknown cause was diagnosed during the early adolescence period in which kidney transplantation was lately performed. His current medications were as follows; everolimus, febuxostat, fluconazole irbesartan, entecavir, Cellcept and atorvastatin.

Mutation analysis of index case and family members

After informed consent, the patient's, parents' and grandparents' genomic DNA was extracted from peripheral blood leukocytes using the DNA Isolation Kit (Qiagen, Valencia, CA). Whole exome sequencing (WES) was performed and revealed a heterozygous missense mutation in *STAT6* which located at coordinate chr12: 57498330C>T for the human genome assembly GRCh37 (hg19) representing nucleotide position 1129, changing G to A (c.1129G>A); (NM_003153.4:c.1129G>A) in the patient and father. This mutation resulted in a change of codon 377 (p.E377K). The mutation was confirmed by sanger sequencing. The variant in the father was *de novo*, suggesting its pathogenicity. Pedigree of the family, the chromatogram of the proband, father and mother and schematic diagram demonstrated the domain organization of human STAT6 were shown in Figure 2A, B and D. Highly evolutionary conserved residue from human to fruit fly was shown in Figure 2C.

Various prediction programs indicate that the mutation is pathogenic. The mutated residue, located in the DNA binding domain of STAT6, presents along the surface of loop regions of the DNA-protein interface (Figure 2E) where STAT6 uses to recognize the palindromic DNA sequences. Switching E to K changes the electric charge of the DNA binding interface from negative to positive charge (Figure 2F) and increase hydrophilicity (Figure 2G), therefore enhances the DNA binding ability of the protein. The mutation is predicted to be damaged by Polyphen-2, M-CAP (predicted possibly pathogenic with score 0.133) and SIFT score. The mutation was absent in 1,080 in-house Thai Exome Database, ExAc database and Wellderly cohort including the 1000 genome database. Finally, *STAT6* variant p.E377K was previously reported in the folliucular lymphoma [(5)], imply that the variant is disease-causing.

Flow cytometric analysis and cytokine measurements from peripheral blood mononuclear cells showed the evidence of exaggerated allergic responses

Flow cytometric analysis of lymphocyte populations (Figure 1E) of the index case revealed increased numbers of T helper type 2 cells, T helper type 17 cells and T helper type 22 cells. T helper type 1 cells were comparable to the controls. Percentages and absolute numbers of T helper populations were shown in Supplementary Table S2. Both class-switched (CD27+) and non-class switched (CD27-) IgE+ memory B cells (CD19+IgD-IgM-IgE+) did not increase (data not shown).

IL-4 and interferon-gamma (IFN-γ) cytokine production from T lymphocytes were measured by flow cytometry (Supplementary Figure S2). Peripheral blood mononuclear cells (PBMC) of the patient, family members, healthy controls and allergic controls were stimulated with PMA (12.5 ng/ml) and ionomycin (I) (0.5 ug/ml). Intracellular staining of IL-4 and IFN-γ were performed. The results were shown as a fold change of mean fluorescent intensity (MFI) compared to unstimulated condition. Our results showed that T lymphocytes of index case secreted IL-4 while cells from family members without *STAT6* mutation and healthy controls had increased IFN-γ production up to three folds, in response to PMA/I stimulation. No IFN-gamma secretion was not observed in the index case and allergic control sample. As we expected, T cells from father did not secrete both IL-4 and IFN-γ since he received immunosuppressive drugs.

IL-4 and IFN-γ production were also measured in culture supernatants of PBMC after stimulated with PMA/I for 24 hours. IL-4 was too low to detect in all samples. Family members without *STAT6* mutation and healthy controls could produce reasonable amounts of IFN-γ; range between 1500-4200 pg/ml. Again, lack of IFN-γ production was found in the patient, father and allergic control.

In summary, all these experiments pointed out that the immune responses of the index case directed toward a type 2 T-helper (Th2) cell phenotype with somewhat lowering a type 1 T-helper (Th1) cell response. Th2 deviation of the index is considerably greater than allergic control who has no *STAT6* mutation since the higher numbers of Th2 cells and IL-4 + T lymphocytes were observed.

Mutant STAT6 preferentially localized in the nucleus

We used immunofluorescence and confocal microscopy to locate STAT6 protein in transfected HEK293T cells, with and without IL-4 treatment (Figure 3). The details of the plasmid and reagents were described in the supplement Methods section. Cells were transfected with STAT6 wide type (WT) and mutant (MU) vectors and stained for detecting the localization of unphosphorylated STAT6 (U-STAT6) and phosphorylated STAT6 (pSTAT6). Without IL-4 stimulation, predominantly cytoplasmic staining with occasionally nuclear signals of the U-STAT6 was found both in WT and MU stat6 transfectants. This is in line with the previous studies [(9)] showing that U-STAT6 can shuttle between the nucleus and cytoplasm independence of stimulation. Detection of pSTAT6 cannot be found in WT and MU at the baseline level. Upon IL-4 stimulation, U-STAT6 in the WT transfectants was equally distributed both in nucleus and cytoplasm while nuclear pattern was predominantly observed in the STAT6 MU. For pSTAT6, even though the nuclear staining was found in both WT and MU transfectants, the signal was much stronger in the STAT6 MU than the WT transfectants.

The similar immunofluorescence findings were described in the somatic mutation of *STAT6* DNA binding domain from follicular cell lymphoma [(5)]. Besides, a strong nuclear band of STAT6 MU was detected at the baseline level using western blot study [(5)]. The different findings between immunofluorescence and western blot might explain by their distinct sensitivity. These experiments point out the characteristic of our STAT6 MU with the nuclear preference, regardless of stimulation.

STAT6 mutant is intrinsically activated, partially independence of IL-4 stimulation

Because STAT6 mutant is nuclear mislocalized and supposed to increase binding affinity to the DNA, we hypothesised that STAT6 mutant should act as a *gain-of-function* mutation. HEK 293T cell luciferase reporter assays are used to measure the ability of STAT6 for binding to its consensus sequence and activate transcription of target genes, with and without IL-4 stimulation. The details of the plasmid, reagents and luciferase experiments were described in the supplement Methods section. The luciferase results were shown in Figure 4A. In STAT6 WT, adding IL-4 leads to the activation of STAT6, as strong induction of DNA binding activity of the STAT6 binding site luciferase response plasmid was observed. For STAT 6 MU, independence of IL-4, a constitutive intrinsic activation was found with ~12 folds higher DNA binding activity compared to mock (n=6 separate experiments). IL-4 stimulation cannot further enhance the transactivation effect of STAT6 MU, assuming that the promotor was saturated or the activation reached the plateau. We hypothesized that this occurred due to the strong binding affinity of STAT6 MU which might preoccupy the promotor area and prevent further binding of the molecule to the DNA.

When we cotransfected STAT6 WT and STAT6 MU in different ratios into HEK 293T cells (n=3 separate experiments) (Supplement figure S3). The results showed that STAT6 MU could counteract the WT transactivation effect in a dose-dependent manner.

Gastric organoids of the patient showed an increase in the allergic responses

Intestinal epithelial cells can secrete a variety of chemokines essential for the chemoattraction of leukocytes in inflammatory diseases. From the histological analysis of patient intestinal cells, dramatic infiltration of eosinophils and mast cells were recognized. From previous studies, following IL-4 stimulation, intestinal epithelium cells could secrete variteis of chemokine to recruit and activate eosinophils and mast cells. To investigate whether intestinal cells from our index case secreting these chemokines in higher degee compared to control, intestinal cells from the gastric biopsy was collected while endoscopy was indicated for protein-losing enteropathy. Controls' biopsies were those whose endoscopy was performed for other reasons such as gastritis and did not have gastrointestinal food allergies or parasitic infections. The stomach organoids of patient and controls were established according to the protocol. Morphology and messenger RNA expression of gastric marker genes were shown in Supplementary Figure S6. Heterozygous *STAT6* mutation p.E377K was confirmed by sanger sequencing. Our result showed that gastric organoids of the patients could spontaneously secrete eotaxin-2 (CCL24) while none of the controls did. A further rising of eotaxin-2 three times higher than controls was detected after IL-4 treatment for 48 hours (Figure 5). Other eosinophil chemotactic chemokines; including monocyte chemotactic protein-3 (MCP3) and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), together with monocyte chemoattractant protein-1 (MCP-1/CCL2), a chemokine selectively recruiting monocytes, neutrophils, and lymphocytes and involving in mast cell degranulation, increased from baseline timepoint in the patient cells without further increase after stimulation. IL-4 was increased only after the stimulation period. Other allergic inflammation-related molecules, including IL-5, IL-10, IL-13, Eotaxin-3 did not differ between patient cells and controls (data not shown).

Discussion:

Monogenic disorders relate to allergy have been recognised for a long time as a part of primary immunodeficiency disorders. Single gene mutation associated with isolated allergic manifestations are rarely described in the past but more identified nowadays [10]. Identifying this disease-identity is crucial due to its strong phenotypic effect, which would allow fundamental insights to the pathogenesis of allergic diseases. Such knowledge opens a great opportunity in developing new targeted therapy for patients with monogenic disorders and also general allergic populations.

In physiologic condition, STAT6-dependent signalling is tightly regulated. It would not be activated without extrinsic signals like IL-4 and IL-13. Our study described, for the first time, a family with *STAT6* mutation in the DNA binding domain. Many lines of evidence showed that the mutated STAT6, both unphosphorylated and phosphorylated forms, owned a spontaneous activity of affinity enhancement and prolonged binding to the DNA. This explains also why it tended to localize in the nucleus. This occurrence results in an increase of signalling proteins involving in the allergic cascades independence of stimulation, such as Th2 cells, eosinophils, mast cells, serum IgE, IL-4 and eotaxin-2. It also fits the phenotypes of the patients whose immediate-type reactions occurred unexpectedly and without clear triggers. STAT6 protein expresses in different types of tissues such as skin, respiratory tissue, intestinal epithelial cells and many kinds of immune cells [11]. Therefore, our patients presented with various allergic symptoms, including atopic dermatitis, allergic eosinophilic gastrointestinal disorders and idiopathic anaphylaxis. As also expected, our patients developed atopic march overtime. Besides, evidence from the mouse model and human diseases demonstrated that aberrantly activated STAT6 in the cyst-lining epithelial cells and tumour cells relate to polycystic kidney diseases and lymphoblastic cancers [5] [6] [12]. This might

explain kidney failure occurred in the father. Long term follows up of the kidney function, and cancer screening in our younger index case should be in consideration.

Nowadays, biologic agents such as anti-IL-4, anti-IL-5, JAK inhibitors are more used as targeted therapies in severe, non-responsive allergic patients. Assuming from the pathway involved, these agents might not work properly in our patients whose STAT6 is spontaneously active. Anti-stat6 molecules should be the more appropriated choice for these particular patients. Screening for the possible single-gene disorders might warrant in the future before starting such expensive biologic agents or even when the patients do not respond well to the treatment.

Indeed, there is no clear indication when monogenic disorders related to allergic diseases should investigate. Our experiences in *STAT6* mutation, together with the studies from other mutation related to allergy [10], suggested that those with early-onset, severe allergic phenotypes and do not respond well to the standard treatment should be targeted. Other comorbidities such as malignancy or somatic features might present depending on the affected genes. This is indeed important since these patients might need a different approach regarding follow-up and treatment.

Unphosphorylated STAT1 and STAT3 elicit a different set of genes expression compared to those activated in response to phosphorylated STAT [13]. While unphosphorylated STAT6 contributed to constitutive cyclooxygenase-2 expression in human non-small cell lung cancer was observed [7], whether different genes are transcripted from unphosphorylated and phosphorylated STAT 6 is yet to be determined.

The association of enamel hypoplasia related to STAT6 overactivation is not clear. Since poor medical health during infancy can affect the formation of enamel or teeth, enamel hypoplasia might be the secondary effect of immune system deviation, as shown in the patients with inflammatory bowel diseases [14]. However, it is worth to note that many primary

immunodeficiency disorders have enamel hypoplasia. Strong enrichment of immune-related cell types was observed among craniofacial tissues [15]. The relationship of enamel hypoplasia and STAT6 should be explored in the future for better understand the link between the immune system and skeletal tissues.

Reciprocal effects of IFN-gamma and IL-4 are well-known in human and mouse models [16]. IFN-γ and IL-4 independently activate STATl and STAT6. However, both STAT1 and STAT6 can bind to STAT-binding element in the IFN regulatory factor-1 (IRF-1) gene and induced IFN-γ-related expression, even though the affinity of STATl is stronger than STAT6. Previous studies suggest that IL-4 may suppress IFN-γ-stimulated transcription through the activation of STAT6, which compete for occupancy of the IRF-1 gene with STATl. Decrease IFN-γ production in our patients possibly resulted from the increase DNA affinity of the mutant STAT6, which might further enhance the suppress of IFN-γ production, in comparison with normal STAT6.

In conclusion, allergic phenotypes can result from more than one mechanism. Besides the known mechanisms of hyperreactivity of involving molecules, our study emphasises the importance of considering the single-gene disorders especially *STAT6* mutation as one of the possible causes in subjects with early-onset severe allergic phenotypes. Identifying mutation enlightened the path to understand the pathophysiology of complex diseases like allergy and lead to a better approach and treatment in the future.

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Figure legends:

Figure 1. Clinical and immunologic phenotypes of the index case.

Erythema and facial swellings occurred in one episode of anaphylaxis (Panel A). Upper endoscopy (Panel B) showed (1) longitudinal furrows with multiple discrete papules on esophageal mucosa and (2) polypoid mucosa of the antrum (yellow arrow). Colonoscopy revealed multiple nodularities and lymphonodular hyperplasia at (3) transverse colon (yellow arrow) and (4) terminal ileum (yellow arrow). Panel C showed the marked eosinophilic infiltration in the lamina propia of the gastric biopsy. Red dots represented eosinophils that infiltrated into epithelium together with reactive epithelial change. Degranulation of eosinophils is diffusely seen. Panel D highlighted numerous mast cells infiltrating in gastric mucosa (red arrow), demonstrated by CD117 immunohistochemistry. Vascular endothelial growth factor (VEGF) (brown staining) was increased in small bowel biopsies (Panel E). Flow cytometry analysis of CD4+ T helper subset using chemokine-receptor surface staining of (Panel F) showed increased numbers of T helper type 2 cells, T helper type 17 cells and T helper type 22 cells of the index case compared to healthy control.

Figure 2. Heterozygous mutation of *STAT6* in the DNA binding domain.

Panel A showed the pedigree of the family. The pedigree contains two affected members (blackened symbols). The index case was determined by the arrow. *STAT6* genomic DNA sequence (Panel B) reveals the c.1129G>A mutation (p.E377K) in proband and his father, but not in the mother. The variant in the father was *de novo*. The mutation is located at an evolutionarily conserved residue presented in different species using the Clustal X program (Panel C). The schematic diagram (Panel D) showed the domain organization of human STAT6, including N-

terminal/alpha-helix domain, DNA-binding domain, Linker domain, SH2 domain (SH2), and Transactivation domain. The red box indicated the mutation in our proband, E377K, located in the DNA-binding domain. Structural modelling of the STAT6CF-DNA complex (Panel E) was downloaded from RCSB protein data bank. Swiss PDB Viewer was used to visualizing the structure and revealed that the *STAT6* mutation is located at the surface of the STAT6 DNA binding region. Switching E to K changes the electric charge of the DNA binding interface from negative to positive charge (Panel F) and increase hydrophilicity (Panel G), potentially resulting in a stronger binding affinity.

Figure 3. **Mislocalization of STAT6 mutant**

Immunofluorescence and confocal microscopy were used to locate STAT6 wide type (WT) and STAT6 mutant (MU); E377K, both unphosphorylated (U-STAT6) and phosphorylated (pSTAT6) forms. The experiments were performed in transfected HEK293T cells with and without IL-4 stimulation. At baseline, both WT and MU stat6 vectors demonstrated predominantly cytoplasmic staining with occasionally nuclear signals of the U-STAT6. Detection of pSTAT6 was not found in both WT and MU. Upon IL-4 stimulation, the distribution of U-STAT6 was found both in the nucleus and cytoplasm of WT transfectants while the nuclear pattern was predominantly detected in the STAT6 MU. For pSTAT6 protein, the nuclear staining was much stronger in the STAT6 MU than in STAT6 WT transfectants. Green, U-STAT 6 & pSTAT6; red, phalloidin; blue, DAPI. IL-4 stimulation is indicated.

Figure 4: STAT6 mutation is intrinsically activating and partially STAT6 independent. Luciferase assay results of STAT6 wide type and mutant in HEK293T cells presented as fold

change compared to empty vector. IL-4 stimulation is indicated. STAT6 mutants demonstrated the autoactivation of luciferase activity in the mutants. The effect could not further enhanced following IL-4 stimulation. W=wide type STAT6, M=mutant STAT6; N4-Luc= STAT6 Luciferase binding site.

Figure 5: Gastric organoids of the index case showed enhanced allergic responses.

Elevated baseline expression with the further increase of eotaxin-2 (CCL24) in stomach oragnoids carrying mutated STAT6 after IL-4 treatment for 48 hours. The expression of eotaxin-2 was measured by Bio-Plex Multiplex immunoassays (Biorad, USA). The mean is indicated.

Figure 6: Illustrated mechanisms of STAT6 mutant links to the clinical phenotypes

Supplementary Figures:

Figure S1: Oral manifestations of index case included enamel hypoplasia (blue arrow), multiple vesicles on tongue and oropharynx (red arrow) and nodules on buccal mucosas (red circle).

Figure S2: Increase IL-4 secretion but lack of interferon-gamma (IFN-γ) cytokine production from T lymphocytes was found in the index case. Fold increase in mean fluorescent intensity (MFI) for IL-4 and IFN-gamma and in T-lymphocytes after 5 hours of stimulation with PMA (12.5 ng/mL) and ionomycin (0.5 ug/mL) were measured by flow cytometric analysis, compared to unstimutated conditions.

Figure S3: Results of STAT6 luciferase assays in HEK293T cells in a different ratio of STAT6 wt and STAT6 mu at baseline and with IL-4 stimulation.

Supplementary Tables:

Table S1: Percentages and absolute numbers of T helper populations of the index case, family members and controls

Table S2: Elevated baseline expression with the further increase of monocyte chemoattractant protein-1 (MCP-1/CCL2), MCP-3, and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), in stomach oragnoids carrying mutated STAT6 after IL-4 treatment. The expression of cytokines and chemokines were done by Bio-Plex Multiplex immunoassays (Biorad, USA).

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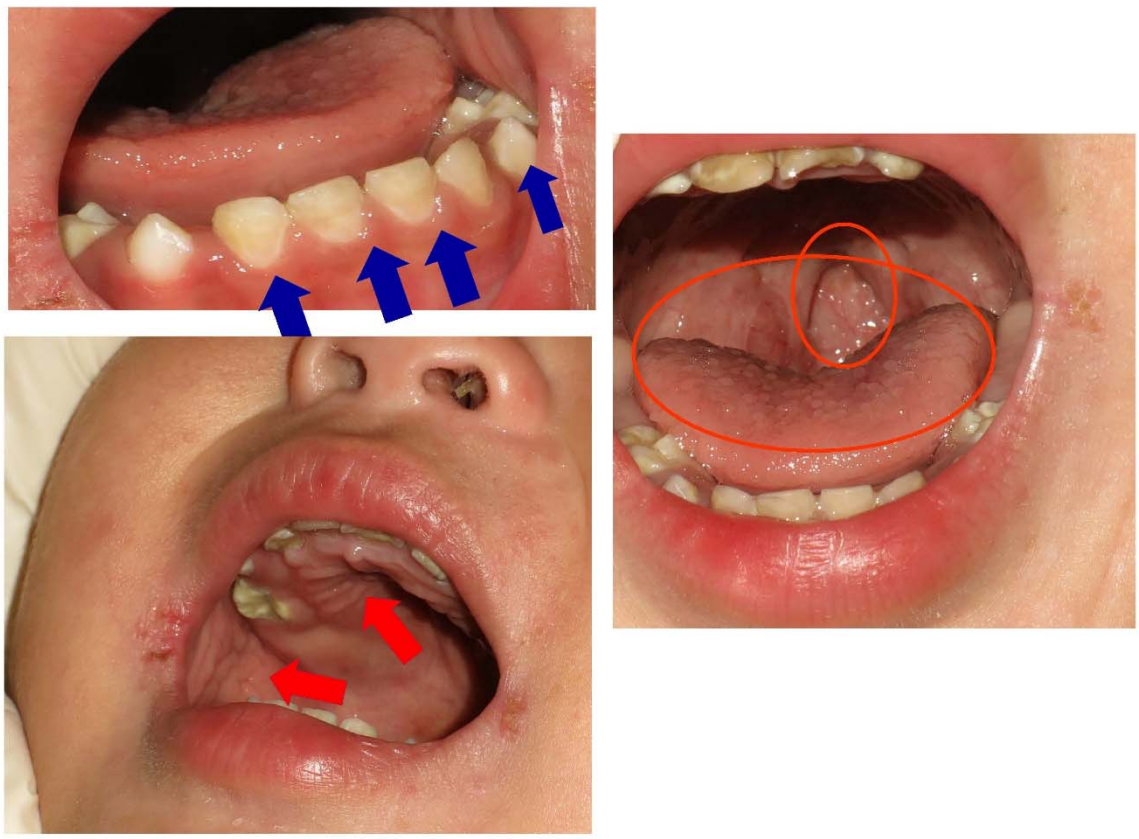
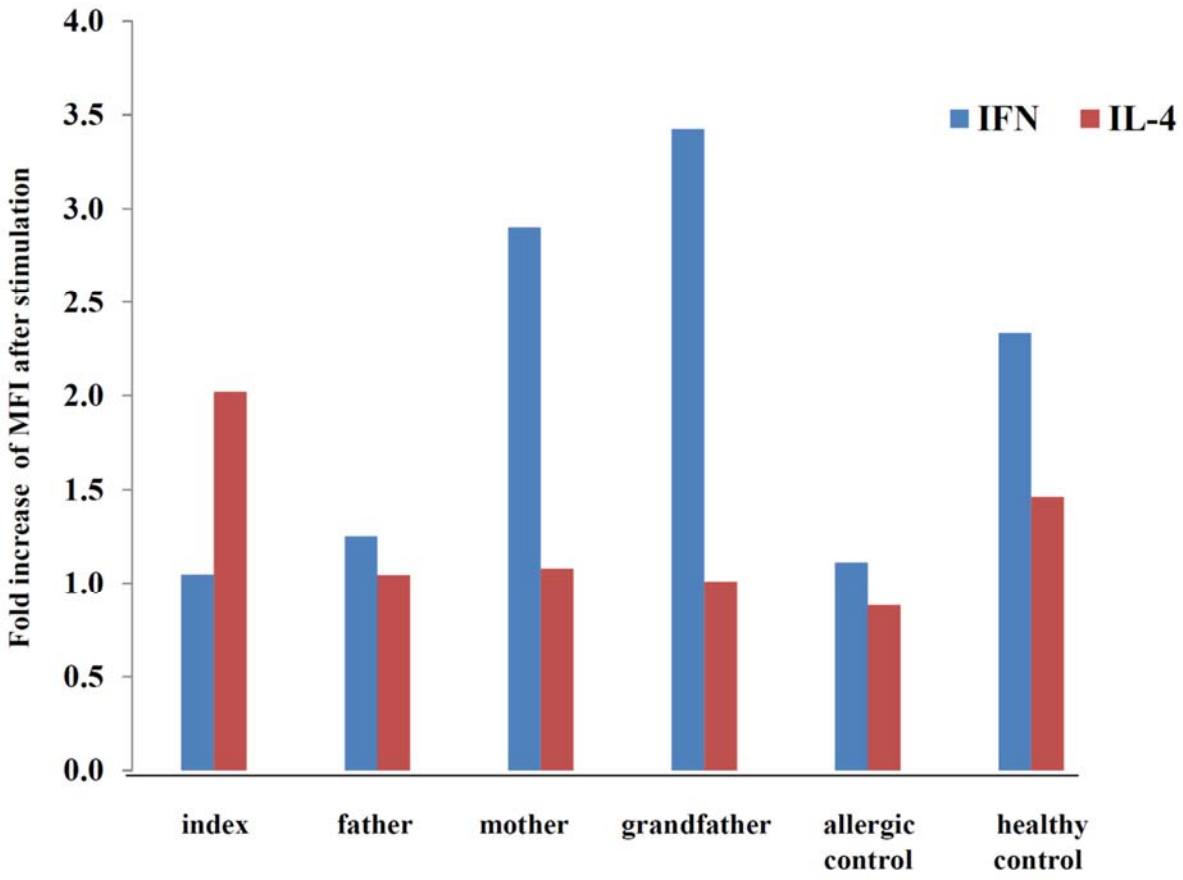
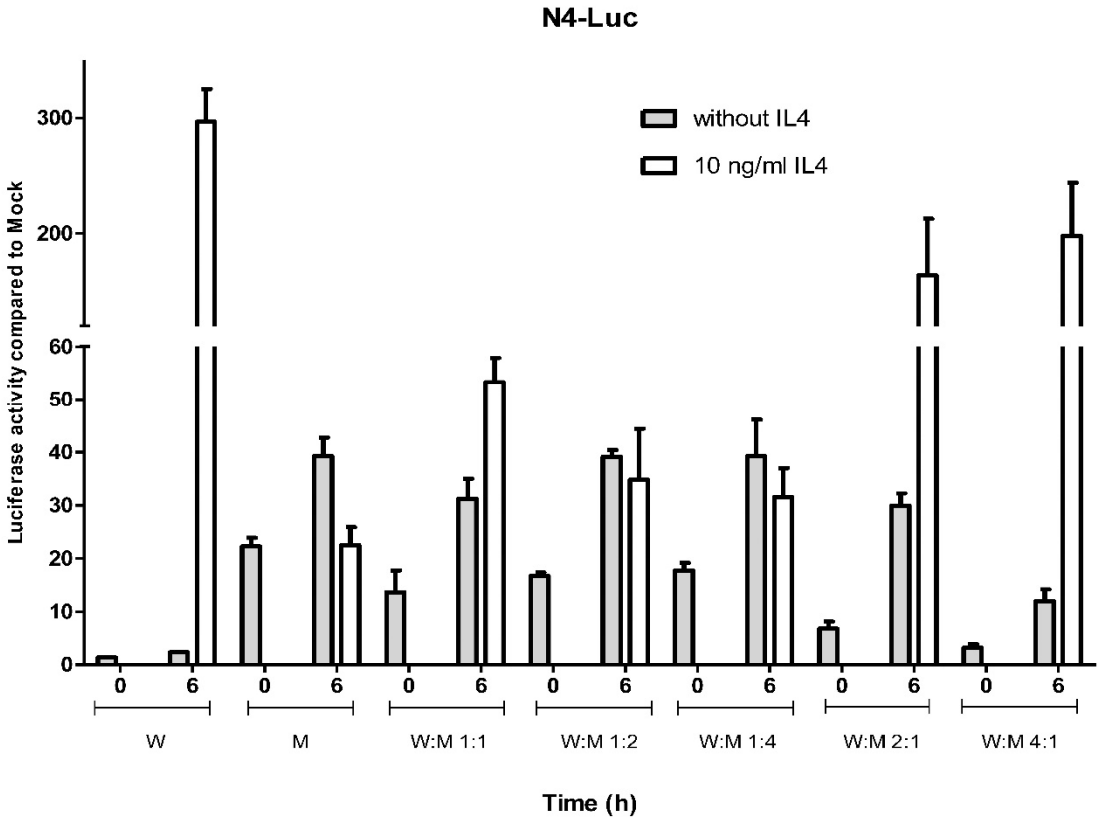
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Table 1. Percentages and absolute numbers of T helper populations

T helper population	Index case	Father	Grandfather	Mother	Control allergic patient
% Th1	0.51	2.4	1.5	1.6	1
absolute number (x 10^6 cells)	33	20	17	21	31
% Th2	4.75	0.8	2.6	0.9	1
absolute number (x 10^6 cells)	314	7	29	12	31
% Th17	1.7	2.3	1.7	1.5	0.5
absolute number (x 10^6 cells)	111	19	19	20	16
% Th22	0.4	0.07	0.7	0.5	0.08
absolute number (x 10^6 cells)	26	1	8	7	3

Supplementary Table 2. Expression of chemokines/ cytokines related to allergic inflammation in stomach organoids carrying mutated STAT6 after IL-4 treatment.

Chemokine / Cytokine (ug/ml)	Index case		Control 1	
	without IL-4	100 ng/ml IL-4	without IL-4	100 ng/ml
MCP-1	607.4	479.94	57.61	42.91
MCP-3	3.48	2.97	UD	UD
RANTES	3.43	3.88	1.01	1.65
IL-4	UD	118.47	UD	UD

MCP-1; monocyte chemoattractant protein-1, MCP-3; monocyte chemoattractant protein-3, RANTES; regulated on Activation, Normal T Cell Expressed and Secreted