



The rs13075270 and rs13092160 polymorphisms of CCR1 and CCR3 genes on oral aphthous-like lesions in PFAPA syndrome

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ABSTRACT

Fever is a common symptom of infection in children. Periodic fever syndromes are less common but more complex. One of these Periodic fever syndromes is PFAPA (periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis) syndrome which is known as the most benign syndromes. The cause of this disease is unknown. Various factors, including environmental and genetic factors, are involved in the development of this disease. In this study, the association of rs13075270 and rs13092160 polymorphisms were investigated in CCR1 and CCR3 genes with susceptibility to this syndrome in the Chinese population. In this regard, 38 patients with PFAPA syndrome and 100 healthy individuals were selected. After DNA sampling and extraction, polymorphisms of CCR1 and CCR3 receptor genes were examined by the PCR-RFLP method. Findings were analyzed using SPSS software version 22 with a significant level of $P < 0.05$. The frequency of T/T genotype rs13092160 polymorphism in the patient and control groups was 78.95% and 83%, respectively, C/T genotype was 21.05% and 17% ($P = 0.421$). The frequency of the C/C genotype was 0 in both groups. Regarding rs13075270 polymorphism, the frequency of T/T genotype in patient and control groups was 15.79% and 81%, C/T genotype was 78.95% and 18% and C/C genotype was 5.26% and 1%, respectively ($P < 0.05$). Thus, in rs13075270 polymorphism, the C/T genotype was associated with the risk of PFAPA syndrome ($P < 0.05$), but rs13092160 polymorphism did not show a significant difference between individuals with PFAPA syndrome and controls.

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Introduction

Fever is a common symptom in the pediatric population. The most common causes of acute fever in children are otitis media and respiratory infections (1). Periodic fever syndromes are associated with greater complexity and are a diagnostic problem for physicians. These syndromes are symptomatic for a few days and then go through asymptomatic periods between attacks (2). Marshall's syndrome or PFAPA (periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis) syndrome was first reported by Marshall et al. in 1987-89 for unknown reasons (3). This syndrome is diagnosed with its classic symptoms, which include periodic fevers (38.5°C - 41°C) for 2-4 days that resolve spontaneously or with treatment (100% of cases), pharyngitis (78% of cases), lymphatic adenopathy (69%), aphthous stomatitis (51%), and arthralgia (33%) (3, 4). It should be noted that other causes of recurrent fevers are ruled out. An adequate response to corticosteroids is a

confirmatory factor in the above syndrome (5).

In this syndrome, other symptoms such as abdominal pain sometimes appear. The onset age of this syndrome ranges from 1 month to 14 years (6). The pathogenesis of the disease is unclear, and the genetic cause has not been determined. It also has no infectious pathogens, so it will not respond to antibiotic treatment (7). There may be mild leukocytosis, increased erythrocyte sedimentation rate, and immunoglobulin. The disease gets better with age. Side effects are rare and the intervals between attacks become longer over time (8). The whole course of the disease varies from 1.5 years to 9.5 years (7). Important differential diagnoses include Hyperimmunoglobulin-D Syndrome (HIDS), recurrent neutropenia, familial Mediterranean fever (FMF), and Tumor necrosis factor receptor-associated periodic syndrome (Traps) (9). Mainly antipyretic drugs and corticosteroids are used in the treatment of the disease. The effectiveness of cimetidine and

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tonsillectomy has been reported in some cases. Out of every 10,000 children in the world, 2.3 are involved with the disease (10).

As mentioned earlier, oral aphthous-like lesions are one of the main symptoms of this syndrome (11). These lesions are a disorder characterized in patients as ulcers confined to the oral mucosa. These sufferers in the complication type of this disease have problems in talking and eating food that reduces the quality of life of these children (7). The clinical presentation is in small round or oval lesions with a definite border, a red halo, and a yellow or gray background. Because of the histological similarities between peptic ulcer and this oral aphthous, it is difficult for specialists to diagnose. Evidence, such as increased levels of immunoglobulins, the presence of circulating antibodies, decreased activity of natural killer cells, and increased secretion of cytokines during inflammation, can help us make a more accurate diagnosis of this syndrome (12).

Chemokines are a family of small-sized inflammatory cytokines that play a significant role in inviting host immune system cells to the site of infection and regulating the movement of leukocytes and other lymphocytes between the site of inflammation and secondary lymphatic organs (13, 14). Chemokines are subdivided into four subfamilies CX, CXC, CC, and CX3X, based on the two cysteine roots near the N-peptide end in their amino acid sequences. Each chemokine binds primarily to several receptors. Chemokines with the CC motif have two cysteines near the amine end placed next to each other without any distance. CCR receptors (CCRs) signal primarily through a membrane protein called G proteins (14). Circulating leukocytes must be removed from the bloodstream to reach the site of inflammation. The slow rate of leukocyte rotation on selectins increases the interaction with chemokines expressed on the suitable surface of the endothelium by glycosaminoglycans (15, 16). The binding of chemokines to their receptors alters the affinity of β -2 integrin, especially CD11b / CD18, on the cell surface of leukocytes and binds to its Ig antigens, such as ICAM. These interactions provide a strong attachment of leukocytes to the endothelium. When leukocytes enter the source of inflammation, a cytokine-rich environment is created that eliminates the influx of antigens (16). CCR3 is a type of CC receptor and contains important ligands such as RANTES and

Eotaxin, MIP-1 α , which is involved in the chemotaxis of mast cells and eosinophils. Important ligands bind to the CCR1 receptor, including macrophage inflammatory protein (MIP-1 α), activation, expression and secretion of natural T lymphocytes (RANTES), and monocyte chemotactic protein-1 (MCP-1) (17).

Also, previous studies have shown a significant relationship between CCR1 and CCR3 polymorphisms of rs13092160 and rs13075270 genes and increased susceptibility to oral aphthous disease (18). Therefore, in the current study, the rs13075270 and rs13092160 polymorphisms of CCR1 and CCR3 genes were investigated on oral aphthous-like lesions in PFAPA syndrome.

Materials and methods

Patients

Because PFAPA syndrome is a rare syndrome (3), our study was performed on 38 PFAPA syndrome children which 16 of them were girls (42.10%) and 22 of them were 22 boys (57.90%) with oral aphthous-like lesions. A specialist evaluated all patients, and inclusion criteria were according to the criteria for diagnosing PFAPA syndrome (4). All children should have these five conditions:

1. Regular recurrent fevers starting at an early age
2. Aphthous stomatitis without respiratory infection
3. No periodic neutropenia
4. Absence of symptoms between periods of fever
5. Normal growth and development

The age of the patients was between 2.3 to 5.7 years old with a mean age of 3.58 years old. The duration of the disease was 6 to 19 months and its average was 10.6 months. At the same time, 100 healthy children without PFAPA syndrome or other autoinflammatory diseases who were geographical, age, and sexually similar to patients and were not related to each other or patients were included in the study as a control group. After giving information, consent was received from the parents of all subjects.

Genotyping

The 2ml of peripheral blood was transferred from the vein of the subjects to tubes containing EDTA (per 1ml, 100 μ l of EDTA 20mM, pH = 8) and stored in a freezer at -20 $^{\circ}$ C until extraction. DNA samples from both groups were extracted with the HigherPurity™ Blood DNA Extraction Kit. The specific primers were

designed to investigate the desired single nucleotide polymorphisms using Gene Runner software (Table 1).

Polymerase Chain Reaction (PCR) was performed in 15µl containing 100ng of genomic DNA, 1.5µl of PCR buffer (1X concentration), 0.5mM dNTP, 1.3mM MgCl₂, 5 pM primer and 1 unit of enzyme *Taq* DNA polymerase (Merck, Germany). PCR program was done by adjusting the optimal conditions for both primer pairs to evaluate the two polymorphisms of CCR1 and CCR3 genes. Denaturation was performed for one minute at 14°C, primer annealing for one minute at 74°C, and extension for one minute at 72°C. These steps were repeated for 30 cycles. The initial denaturation was 5 minutes at 94°C and the final extension was 10 minutes at 72°C. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide under UV light. Genotypes were determined using the PCR-RFLP technique. The RFLP reaction consists of 5µl of PCR product, 0.2µl of limiting enzyme, 1µl of suitable buffer and 3.8µl of distilled water. The samples were incubated at 37°C for 24 hours. PCR products were separated by the restriction enzymes on polyacrylamide gel electrophoresis and after staining with silver nitrate, the results were evaluated.

Table 1. Association between the expression level of SOX2 and mTOR to characteristics of various clinical cases; Name of Polymorphism (A), Enzyme (B), Primer Sequence (C), PCR Product Length (D)

A	B	C	D
rs13092160	Mnl1	Forward 5'- ATTACCTTTAT GTGGCTGCTCAT	252 bp
		Reverse 5'- TCCAATCAGCTA GATAGACATGAC	
rs13075270	Mva1	Forward 5'- TGTGAGATGTCC CTGTTGCCTAAT	271 bp
		Reverse 5'- TGGGGCACACTG TCTGCATGACGA A-3'	

The PCR product for rs13092160 polymorphism has a length of 252 bp. Enzymatic cleavage with Mnl1 in individuals with C/C genotype produces three pieces of 56, 94 and 102 bp, in individuals with C/T genotype four pieces of 56, 94, 102, and 196 bp, and

individuals with the T/T genotype, two fragments, 56 and 196 bp.

The PCR product of rs13075270 polymorphism had a length of 271 bp. Mva1 enzyme cleavage in individuals with C/C genotype produces four fragments of 44, 63, 65, and 99 bp, in individuals with C/T genotype produces five fragments of 44, 63, 65, 99, and 109 bp, and in individuals with T/T genotype produces three pieces 63, 99, and 109 bp.

Statistical analysis

Statistical analysis of data was performed by SPSS statistical software (version 22). The distribution of genotypes for each polymorphism and allelic frequency in the two groups of patients and control was calculated based on Hardy-Weinberg equilibrium and was measured by the chi-square test. Also, for each polymorphism, the odds ratio (OR) was calculated for both control and ill groups with a 95% confidence interval. A P-value of less than 0.05 was considered a significant level.

Results and discussion

The genotypic and allelic distribution of rs13092160 polymorphism showed that there were no significant differences between the control group and patient group about genotype ($P=0.421$) and allele ($P=0.872$) (Table 2). It should be mentioned that the CC genotype was not observed among both groups.

Table 2. The genotypic and allelic distribution of rs13092160 polymorphism

Genotype	Control Group (n=100)		Patient Group (n=38)		P-value
	Number	Percent	Number	Percent	
T/T	83	83	30	78.95	0.421
T/C	17	17	8	21.05	
C/C	0	0	0	0	
Allele	Number	Percent	Number	Percent	0.872
T	92	92	35	92.10	
C	8	8	3	7.90	

Despite rs13092160 polymorphism, the genotypic and allelic distribution of rs13092160 polymorphism showed that there were significant differences between the control group and patient group about genotype ($P=0.001$) and allele ($P=0.001$) (Table 3).

Table 3. The genotypic and allelic distribution of rs13075270 polymorphism

	Control Group	Patient Group	P-value
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Genotype	(n=100)		(n=38)		
	Number	Percent	Number	Percent	
T/T	81	81	6	15.79	0.001
T/C	18	18	30	78.95	
C/C	1	1	2	5.26	
Allele	Number	Percent	Number	Percent	0.001
T	90	90	21	55.26	
C	10	10	17	44.74	

PFAPA (periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis) syndrome is one of the most benign syndromes. Almost all pediatricians and infectious disease physicians encounter children's parents who complain of recurrent fever and are always diagnosed as permanent sick children by their parents (3). PFAPA syndrome in these children is always presented as a possible diagnosis (19). PFAPA has two prominent characteristics: the first is periodic sudden fever (usually four weeks), lasting 3-6 days, and no respiratory or other specific symptoms; the second characteristic is that the patient is completely normal in the intervals of attacks and has completely normal strength and energy, and with frequent fever attacks, the patient has good growth criteria (4).

Many of these children have been diagnosed with tonsillectomy and streptococcal pharyngitis, but all laboratory tests of these patients are normal and cannot be ruled out (19). The serological, immunological, and hematological examinations were completely regular in these patients and did not show any specific findings (20). PFAPA exists in all genders, races, and regions. The age of onset is usually under five years old and may last into adolescence and eventually subside on its own. PFAPA does not cause any side effects (21). The use of steroids on the first day of fever can improve the patient's symptoms, so diagnosing an unexplained disease may involve an exacerbated inflammatory process. There are currently six criteria for diagnosing PFAPA, including frequent recurrent fever (starting in children under five years of age), general symptoms without respiratory symptoms, and at least one oral plaque, pharyngitis, or cervicitis during seizures, elimination of periodic neutropenia, the asymptomatic person at intervals of fever attacks, good growth of the patient despite recurrent fever, and the excellent response to corticosteroids is at the beginning of the disease (22).

In this study, we tried to evaluate PFAPA syndrome by considering the polymorphism of CCR1 and CCR3

genes. Various polymorphisms have been reported in the CCR3 and CCR1 genes associated with multiple diseases, including PFAPA syndrome (18). Examples are rs13075270 and rs13092160 polymorphisms. There are minimal studies on the association of rs13092160 polymorphism with this syndrome. The polymorphism (rs13092160) C/T is located on chromosome 2 at the nucleotide position 47202211 (23). This polymorphism is located in the intron region, and its clinical significance is unknown. The mechanism and mode of action of this polymorphism have not been studied (3).

In the present study, for rs13092160 polymorphism, the homozygous T/T genotype was the highest in both patient and control groups. According to the results of the chi-square test, the *P*-value for allelic distribution was equal to 0.872 and greater than the value of 0.05, which indicates that there is no significant relationship between rs13092160 polymorphism and the possibility of PFAPA syndrome.

Limited studies have been performed on polymorphism (C/T) rs13075270 (24). The rs13075270 polymorphism is located on chromosome 2 at the nucleotide position 47202211. This polymorphism is located in the intron, and its clinical significance is unknown. So far, no study has been done on the mechanism and function of this polymorphism (25-27). The present study results show that in the patient group, the frequency of heterozygous C/T genotype and the control group, the frequency of homozygous T/T genotype is higher than other genotypes. There was a significant difference between rs13075270 polymorphism and susceptibility to PFAPA syndrome ($P = 0.00031$). Therefore, it can be concluded that the presence of C/T genotype is a predisposing factor for this disease.

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