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Association of REL Polymorphism with Cow's Milk Proteins Allergy in Pediatric Algerian Population. Association REL Polymorphism with Cow's Milk Proteins Allergy

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ABSTRACT

Introduction: Cow's milk proteins allergy (CMPA) pathogenesis involves complex immunological mechanisms with the participation of several cells and molecules involved in food allergy. The association of polymorphisms in the interleukin 4, Forkhead box P3 and the avian reticuloendotheliosis genes was investigated in an infant population with CMPA of Western Algeria. **Materials and methods:** We obtained DNA and clinical data from milk allergic subjects during active phase and from a group of non-atopic control subjects. **Results:** Our findings showed that the allele G of the cRel gene intronic polymorphism at +7883 positions was significantly higher among cow's milk proteins allergic patients compared to control subjects. **Conclusion:** The results of this study suggest a possible association of CMPA with cRel G+7883T polymorphism.

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Introduction

Cow's milk proteins allergy (CMPA) is defined as any adverse-reaction-mediated immunological mechanisms against one or more milk proteins (1). The symptoms of milk allergy that appear 2 h after ingestion of milk are due to IgE- and mast-cell-mediated reactions (2,3). This allergy appears at an early age, in fact, 82.5% of CMPA infants developed it in the first 3 months of life (4). This is because the majority of patients, with severe food allergies, are atopic and 10% of them have other symptoms of atopy (5). If one parent is allergic, the risk of allergic reactions in children would be 30–40% (6). If both parents are affected, the risk would increase (70–80%).(7).

In Algeria, the prevalence of IgE-mediated CMPA is estimated at 1.1% (8). However, the prevalence in the Spanish population (Valencia community) (9) indicates a lower prevalence of IgE-mediated CMPA; 0.4%.

Allergies involve the participation of components of the innate and adaptive immune responses, such as mast cells, basophils and eosinophils, as well as activated T helper 2 (Th2)

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cells and B cells types, switched to the IgE production. Immune regulatory mechanisms, that normally operate to maintain the allergen tolerance, breakdown for reasons that still remain unknown (10).

One of the most important cytokines in the Th2 pathway is the interleukin-4 (IL-4), a member of the α -helical cytokine family, the later is produced by activating CD4+ T cells, basophils and mast cells. IL-4 is important in the B-cell activation and differentiation into plasma cells, it induces class switching to IgE. In addition, IL-4 plays an important role in T-cell proliferation and in inhibiting Th1 cell differentiation. Several epidemiological and genetic studies demonstrated the association of the polymorphisms in the IL4 gene and several allergies such as allergic asthma or allergic rhinitis (11). The study by Rautava and Isolauri (12) reports that CMPA children have a higher production of IL-4.

So far, several studies have given great interest to IL4-33C/T polymorphism with different types of allergy like asthma (13,14). However, not enough studies were oriented to explore this association with CMPA. Likewise, studies have shown that asthmatic patients with the -33T allele had higher levels of IL-4 in their serum than those with the -33C allele (15). These results led us to say that the -33C/T polymorphism could influence the risk of food allergy occurrence by affecting the level of IL-4 expression.

It has been reported that this single nucleotide polymorphism (SNP) is associated with other types of allergies (16–19). A meta-analysis of Hai-Jun Yang (20) demonstrated that this polymorphism constituted a major risk factor for asthma. The studies conducted in Australia (21), Japan (13,22), Canada (23), the United States (24), Finland (25), the United Kingdom (26,27), China (28–30) and Taiwan (31) found no significant association between -33 C/T and asthma. No genetic and immunologic evidence reinforces the idea of a central role for regulatory T (Treg) cells in preventing pathological immune responses, including autoimmunity, inflammation and allergy by promoting tolerance to self and environmental antigens. Tregs cells express the forkhead box P3 (FoxP3) transcription factor (32). FoxP3 plays a pivotal role in oral tolerance. The consequence of deficiency or dysfunction of Tregs is the subject of several studies, therefore the importance of exploring the FoxP3 gene (33).

In 2016, Papare et al. demonstrated through an epigenetic study that the expression of FoxP3 in children with IgE-mediated CMPA was considerably decreased compared to children becoming tolerant and controls (34). Therefore, following the number of studies carried out on the association of this polymorphism with the other types of allergies, FoxP3 promoter polymorphism A/C at position -3279 appeared obvious to us (35).

In the continuity of our work on the Treg cells, we are interested in the avian reticuloendotheliosis (c-Rel) transcription factor. The c-Rel is a subunit of the mammalian Rel/nuclear factor (NF)- κ B family (NF- κ B), which has an inhibitory transactivation domain (36). It regulates the expression of different genes in the B and T lymphocytes that are crucial for cell division and immune functions. c-Rel controls the balance between tolerance and immunity by specifically regulating certain genes encoding proteins involved in immune functions such as IL-2 and FoxP3 (37). c-Rel was found to induce FoxP3 transcription directly by binding to regulatory elements in the FoxP3 gene (38). In 2009, Long et al. (39) suggested that c-Rel binds to a distal FoxP3 region to the promoter named conserved non-coding sequence 3 (CNS3) for the FoxP3 transcription, from where the importance to study this gene.

Several human studies have demonstrated the crucial role that this gene may have on the inflammatory balance (40), celiac disease (41), B lymphoma (42), psoriasis (43), rheumatoid arthritis (44) and ulcerative colitis (45). More specifically, the intronic SNP G+7883T

in this gene was associated with susceptibility to rheumatoid arthritis (36) and psoriasis (46).

To our knowledge and to the present time, no study has been carried out on the implication of these polymorphisms with food allergies, even less on the CMPA.

Material and methods

Population study

The sample was divided into two groups: control subjects without any allergies and subjects with IgE-mediated CMPA confirmed by an oral challenge and clinical history. This case-control study includes 58 unrelated subjects of Algerian origin (between 1 month and 4 years old) and consisted of 30 cases and 28 healthy controls. The subjects were recruited from gastroenterology and nutrition service of a specialized pediatric hospital center in Oran. They have signed a consent form after being informed of the nature of the study. All cases had positive specific IgE (sensitized against one or more milk allergens).

The ethics committee at the National Evaluation and Planning Committee of the Algerian University Research approved this study objectives and procedures.

Patients with chronic medical conditions, especially autoimmune diseases were excluded. Pediatric gastroenterology specialists performed the clinical evaluation for allergies. Specific IgE was assessed by Enzyme Linked Immuno Fluorescent Test against milk proteins. A complete questionnaire on the family history and the history of the disease was submitted to the parents of the subject. The inclusion criteria required that all subjects must be in good general health and do not have any manifestation of autoimmune diseases or any other type of allergies. The patients classified as allergic were diagnosed according to the criteria of the World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines (47).

All controls were voluntary, they gave their blood for an IgE specific testing for cow's milk proteins, DNA analysis and enlightened consents were signed. These healthy controls did not have atopy, milk allergy or a family history of milk allergies. Any suspicion in the clinical classification was excluded from the study.

Finally, the explorations for both allergic patients and healthy controls included the clinical history. The prick test was considered positive when the wheal diameter was greater than 3 mm, specific IgE with a positive cut-off above 0.35 kU/L to cow's milk proteins. Oral challenge was performed by administering increasing amounts of fresh pasteurized milk infant formula in 20-min intervals to the usual dose starting from 0.1 mL until the first symptoms appeared. This was performed under close medical supervision.

DNA extraction

A total of 2–5 mL whole blood samples were collected from each participant in Ethylène Diamine Tétra-Acétique (EDTA) tubes. Genomic DNA was isolated from peripheral blood leukocytes by means of the Stratagene kit (Agilent Technologies, USA). The DNA samples were stored at -20°C in TE buffer (10 mM Tris, 1 Mm EDTA) until required. The concentration and the purity of DNA were assessed by the absorbance ratio at 260 nm/280 nm. DNA integrity was checked and quantified using agarose gel electrophoresis.

Table 1. Genotyping parameters.

SNPs	Taqman assay	Context sequence [VIC/FAM]
IL4 (+33C/T)	C__16176215_10	TTAGCTTCTCTGTATAAACTAATTG[C/T]CTCACATTG TCACTGCAAATCGACA
FoxP3 (−3279A/C)	C__27476877_10	GGTGCTGAGGGGTAACTGAGGCCT[T/G]CAGTT GGGGAGAGAGCCAGAACCAG
REL (+7883G/T)	C__3219755_10	TAAAGTTTGAAAAATGGCTCATGT[G/T]TACTTC ATTGTCCTTTCTTTATTGC

Genotyping

All samples were genotyped on TaqMan real-time polymerase chain reaction (PCR) (Applied Biosystems (ABI), Foster City, CA, USA). The test reaction was performed in a 20 μ L mixture containing 1X of TaqMan Assay, TaqMan Genotyping MasterMix and DNA per well. A negative control was included in all wells (well without DNA). The assay includes two locus-specific PCR primers that flank the SNP of interest (F forward and R reverse) and two allele-specific oligonucleotide TaqMan fluorescent probes (VIC and FAM) (Table 1).

Statistical analysis

The Hardy–Weinberg equilibrium was applied using Pearson's χ^2 test for controls with a degree of freedom. The association between IL4(C-33T), FoxP3 (A-3279C) and cRel (G + 7883T) polymorphisms and CMPA risk were calculated as odds ratio (OR) with 95% confidence intervals (CI). The differences were considered statistically significant when $p < 0.05$. All statistical analyses were performed by IBM SPSS Statistic 23 version (SPSS Inc., Chicago, Illinois).

Results

Patients and controls characteristics

Patients and healthy controls characteristics are shown in Table 2. The average age was 15.2 months in the patients group versus 9.3 months in the control group. There was no statistical difference between CMPA patients and controls in terms of gender, age, bloating, oesophagitis, rectocolitis and constipation. However, there was a significant difference with respect to the other characteristics.

Table 2. Baseline characteristics of all the subjects.

Parameters	Case (n = 30)	Control (n = 28)	p value
Gender, F/M	9/21	14/14	NS
Age (month)	15.2	9.3	NS
Diarrhea	22	16	0.001
Vomiting	19	6	0.001
Colic	10	2	0.014
Bloating	4	3	NS
Gastroesophageal reflux disease	8	0	0.003
Oesophagitis	2	0	NS
Rectocolitis	1	0	NS
Enterocolitis	4	0	0.045
Constipation	3	3	NS

NS: Non-statistical difference.

Distribution analysis of IL4, FoxP3 and c-REL frequencies between cases and controls

All samples were analyzed for polymorphisms of the IL4 (−33 C/T), FoxP3 (−3279 A/C) and cRel (+7883G/T). The genotype distributions of these polymorphisms in controls group were consistent with the assumption of Hardy–Weinberg equilibrium ($p > 0.05$, $\chi^2 < 3$) (Table 3).

Frequencies distribution of IL4 (−33 C/T) and FoxP3 (−3279C/A) genotypes and alleles

Concerning the case group, the homozygosity for the C allele at position −33 of IL4 was noted in 64.28% compared to 78.58% of healthy subjects and homozygosity for T allele in 7.14% versus 0% of healthy subjects (Table 3). In addition, homozygosity for the T allele at position −3279 of FoxP3 was noted in 42.85% compared to 37.03% of healthy subjects and homozygosity for G allele in 25% compared to 48.14% of healthy subjects (Table 3).

Thus, we found no significant differences in the genotype distribution of both IL4 (−33C/T) and FoxP3 (−3279A/C) polymorphisms between cases and controls, (OR [95% CI] = 0.19 [1.11–6.20], $p = 0.26$ and OR [95% CI] = 0.79 [1.11–6.20], $p = 0.14$, respectively).

The same result was found in allelic distribution to the allele T of IL4 (OR [95% CI] = 0.45 [1.03–2.49], $p = 0.14$) and the allele A of FoxP3 (OR [95% CI] = 0.56 [1.03–2.49], $p = 0.12$) (Table 3).

Frequencies distribution of cREL (+7883G/T) genotypes and alleles

The homozygosity for the G allele was noted in 57.14% of cases compared to 14.81% of healthy subjects, and homozygosity for T allele was noted in 9.52% of cases compared to 59.25% of healthy subjects. These differences are significantly different (OR [95%CI] = 11.19 [2.45–51.00], $p = 0.001$) (Table 3). Furthermore, significant association in allelic distribution to the allele G was found (OR [95%CI] = 4.11 [1.82–9.29], $p = 0.0004$).

Table 3. The genotype and allele frequencies of IL4 (C+33T), FoxP3 (A-3279C) and REL (G+7883T) polymorphisms in CMA patients and healthy controls.

Polymorphisms		CMA patients, $n = 30$ (%)	Controls, $n = 28$ (%)	χ^2	p value	OR (95% CI)
IL4	Genotypes	$n = 28$	$n = 28$	2.68	NS	0.19
	C/C	18 (64.28)	22 (78.58)			
	C/T	8 (28.57)	6 (21.42)			
	T/T	2 (7.14)	0 (0)	2.38	NS	0.45
	Allele					
	C	44 (78)	50 (9)			
FoxP3	T	12 (22)	6 (10)	3.888	NS	0.79
	Genotypes	$n = 28$	$n = 27$			
	T/T	12 (42.85)	10 (37.03)			
	T/G	9 (32.14)	4 (14.81)	2.31	NS	0.56
	G/G	7 (25)	13 (48.14)			
REL	Allele			14.363	0.001	11.19 [2.45–51.00]
	T	33 (59)	24 (44)			
	G	23 (41)	30 (55)			
	Genotypes	$n = 21$	$n = 27$	12.326	0.0004	4.11 [1.82–9.29]
	G/G	12 (57.14)	4 (14.81)			
	G/T	7 (33.33)	7 (25.92)			
	T/T	2 (9.52)	16 (59.25)			
	Allele					
	G	31 (74)	15 (28)			
	T	11 (26)	39 (72)			

χ^2 : khi-deux de Pearson, NS: No statistical difference, OR: Odds ratio.

Discussion

The aim of the present study was to assess, among the specific western Algeria pediatric population, the association of these three risk gene polymorphisms with CMPA.

The dataset description is contrary to Boughellout and Zidoune's study (48) in an eastern Algeria population, where girls were found to be much more affected than boys. Our study, carried out in the western Algeria population, showed no significant difference in terms of gender and CMPA.

Diarrhoea, vomiting, colic, gastroesophageal reflux and enterocolitis were the most frequent manifestations. This was confirmed by the preliminary studies on the Danish, Norwegian, Finnish, British and German populations (49–54).

Until now, there are few studies that were interested in the genetics of CMPA. However, several association studies have explored the role of certain genes such as IL4, FoxP3 and cRel in the occurrence of other types of allergies like asthma, allergic rhinitis and food allergy (55–57).

In the current study, the association between three different SNPs in three different genes IL4 (–33C/T), FoxP3 (–3279A/C), cRel (+7883G/T) and CMPA in pediatric Algerian population were investigated. Results showed that no statistically significant differences were evident between cases and controls in terms of the allelic and genotypic distributions of IL4 –33C/T and FoxP3 –3279A/C polymorphisms. It seems that these two polymorphisms do not contribute to the risk of CMPA. If this result is confirmed by other studies or by population widening, this would indicate that the polymorphisms IL-4 –33C/T and FoxP3 –3279A/C are not implicated in the CMPA development.

A clear significant association was obtained between CMPA and cRel intronic polymorphism +7883G/T ($p = 0.001$). Moreover, the presence of at least one copy of the G allele showed a significant difference between CMPA patients and healthy subjects (GG+ GT/TT, $p = 0.00001$; OR = 11.19 and 95% CI [2.45–51.00]); (GT/TT, $p = 0.01$) (data not shown).

The G allele cRel proved to be significantly associated with CMPA in our pediatric population with a significant difference ($p = 0.0004$). Contrary to the previous studies, where allele G was considered as the major allele and was shown to have a protective effect, in our population it conferred a statistically significant ($p < 0.05$) risk for this pathogenesis. This may be another instance of the so-called “flip-flop phenomenon” presumably due to variations in linkage disequilibrium with the causative alleles in different populations (58). Our results corroborate the study of Bekada et al. (59); the authors demonstrated that the Algerian population is genetically heterogeneous and different compared to the other populations and this could explain the pathogenic role of the cRel G allele in the case of CMPA compared to other populations.

Future studies, using an even larger number and potentially more ethnically diverse CMPA samples, may be needed to clarify definitely the role of the cRel (+7883G/T) in CMPA pathogenesis.

There is no element of comparison with another study of the association between this polymorphism and CMPA. The only way to confirm this result would be to reproduce the study on a wider panel.

Structural and functional effects of this polymorphism remain unknown and need to be more investigated. Due to the intronic location of this polymorphism, it is possible that this affects the transcriptional efficiency of cRel gene, consequently affecting the function of FoxP3.

This is the first and the only study to explore the association of the IL-4, FoxP3 and cRel genes with CMPA in the pediatric population, where genotype and allele frequencies were established. We were interested in the IgE-mediated form of CMPA. The CMPA was studied and compared to healthy controls from the same population. Furthermore, the subjects of this study were selected from a homogeneous population with similar ethnic and socioeconomic status. We propose to increase the sample size to confirm our findings of the non-association of the two polymorphisms –33 C/T IL4 and –3279C/A FoxP3. Whereas it can be concluded that cRel gene polymorphism could trigger the disease, cRel genotype appears to be an important risk factor for CMPA in this population. Therefore, it will be necessary to explore other genes that are involved in the process of tolerance.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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