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Polymorphism of *IL10*, *IL4*, *CTLA4*, and *DAO* Genes in Cross-Reactive Nonsteroidal Anti-inflammatory Drug Hypersensitivity

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Abstract

Our aim was to evaluate genetic polymorphism of molecules involved in immunoregulatory/allergic processes in patients who presented with cutaneous hypersensitivity caused by chemically unrelated nonsteroidal anti-inflammatory drugs. Polymorphisms at *IL10* (−1082 G>A), *IL4* (−589 C>T), *CTLA4* (+49A>G), and *DAO* (+8956 C>G) genes were studied in 55 cases and 97 controls by the polymerase chain reaction-restriction fragment length polymorphism technique. With regard to the polymorphism at *IL10* −1082, higher frequencies of the AG genotype (57% vs 39%) and G allele carriers (70% vs 48%) were found among the patients, indicating a risk effect (odds ratio [OR] = 2.56 and $P = .01$ for AG genotype and OR = 2.52; $P = .01$ for AG/GG). For the *CTLA4* +49 A/G single-nucleotide polymorphism (SNP), AG genotype (31.0%) ($P = .02$) and G carrier (54.0%) ($P = .05$) frequencies were found to be significantly lower in the patient group compared with the control group (51.0% and 69.0%, respectively). The SNP *DAO* +8956 C>G was associated with a strong protective effect, with OR values of 0.83 for CG and 0.11 for GG genotype ($P = .04$ for the codominant model), suggesting an allele dose effect. The combination of *IL10* and *DAO* SNPs in a multivariate model did not alter the OR values, suggesting independent effects for both SNPs. The results are striking. In conclusion, these results suggest that polymorphisms in regulatory targets of the immune response and in *DAO* gene could modulate an individual's susceptibility to nonsteroidal anti-inflammatory drug hypersensitivity reactions. Further studies will be necessary to complement our results.

Keywords

NSAIDS, drug hypersensitivity, genetic polymorphisms, cytokines

According to the World Health Organization, over the last few decades, morbidity and mortality associated with drug use have been considered a major public health problem throughout the world.[1] Adverse reactions to nonsteroidal anti-inflammatory drugs (NSAIDs) affect 0.5% to 1.9% of the general population and account for about 20% to 25% of all adverse reactions.[2] The main causative drugs are NSAIDs, β-lactam antibiotics, sulfonamides, contrast agents, and antiretrovirals.[3,4] The frequency of hypersensitivity reactions to NSAIDs may vary according to the population studied, the diagnostic method used, and the type of clinical manifestation.[5] In many countries some of these drugs (eg, acetylsalicylic acid, paracetamol, ibuprofen) are sold over the counter. For this reason irrational use of NSAIDs is a frequent cause of adverse events.[6–8] Hypersensitivity reactions to NSAIDs include nonallergic and allergic reactions.

Urticaria and/or angioedema induced by multiple NSAIDs occurs after the administration of nonstructurally related NSAIDs in the absence of baseline respiratory or cutaneous diseases.[9,10] It is hypothesized that the main mechanism enrolled in this category is inhibition of COX-1.[2]

In the last decade much attention has been paid to genetic factors as predisposing and triggering fac-

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tors of hypersensitivity reactions.[8,11] Research on the genetics of hypersensitivity to NSAIDs has increased dramatically, particularly for respiratory diseases exacerbated by NSAIDs and for chronic urticaria exacerbated by aspirin.[11,12] Nonetheless, there are few studies regarding acute urticaria induced by NSAIDs.[8] Genetic polymorphisms that increase or dysregulate the expression of genes involved in the production of cysteinyl leukotrienes[8,11,13,14] or in the metabolism of the histamine[15] may be considered risk factors for hypersensitivity induced by various NSAIDs. Ayuso et al[15] found that the +8956C/G polymorphism and consequent His645Asp substitution are responsible for the decrease in the enzymatic activity of diamino oxidase. The enzyme metabolizes histamine, and a decrease of its activity would lead to more histamine available in the milieu.

Although it is suspected that mechanisms that lead to hypersensitivity to multiple NSAIDs are not allergic, there is some evidence in the literature that shows that there may be some contribution of immunological mechanisms, either by increasing or decreasing the risk.[16] In the present work we evaluate the polymorphism of molecules generally involved in immunoregulatory processes (eg, cytotoxic T lymphocyte antigen [CTLA]-4 and Interleukin [IL]-10) or in allergic manifestations (eg, IL-4) and in the metabolism of histamine (ie, diamino oxidase) in a case-control study comparing patients who presented cutaneous hypersensitivity caused by nonstructurally related NSAIDs and those who were tolerant to these drugs.

Methods

The study was approved by the Research Ethics Committee of the Walter Cantídio University Hospital in Fortaleza, Ceará, Brazil, under approval number 550.608. The work was a case-control study. All the participants (patients and controls) were invited to sign an informed consent form.

Subjects

Patients aged 18 to 80 years (n = 55) were selected at the Dermatology Outclinics, Walter Cantídio University Hospital/Federal University of Ceará. The patients experienced as least 2 episodes of urticaria and/or angioedema in their lifetime with 2 or more chemically unrelated NSAIDS including strong COX-1 inhibitors. Clinical evaluation was done by an allergist. According to Romano et al,[17] patients who present adverse reaction against unrelated NSAIDs should not be rechallenged unless probable causative factors such as food might also be suspected. NSAID-tolerant controls, matched by age and sex, were selected at a blood bank, HEMOCE (Hemocentro do Ceará). The controls comprised individuals who take NSAIDs for a long time without experiencing any episode of hypersensitivity reactions to the drugs. After the project was explained and informed consent was obtained, a questionnaire was applied. Pregnant women were excluded from the study. Patients taking β-blockers or angiotensin-converting enzyme inhibitors, and those with severe infections or with underlying heart disease were also excluded from the study.[9,18]

It is a consensus in the literature that NSAID-exacebated respiratory/cutaneous diseases and urticaria and/or angioedema induced by NSAIDs probably share similar nonallergic mechanisms, that is, overproduction of leukotrienes. Therefore, skin tests are not recommended for these groups, and provocation tests could be performed to search for safe therapeutic alternatives.[5,19] At the end of the clinical evaluation, the patients were instructed to avoid strong COX-1 inhibitors.

DNA Extraction

Genomic DNA was extracted from whole individual blood samples collected in EDTA tubes. DNA extraction was performed using the commercial kit HiPurA™ Multi Sample DNA purification (Himedia Laboratories, India) according to the manufacturer's recommendations.

Single Nucleotide Polymorphism Genotyping of *IL10* (–1082 A>G), *IL4* (–589 C>T), *CTLA4* (+49 A>G), and *DAO* (+8956 C>G)

Genetic polymorphisms of *IL10* (–1082 A>G), *IL4* (–589 C>T), *CTLA4* (+49 A>G), and *DAO* (+8956 C>G) were analyzed by the polymerase chain reaction (PCR)-restriction fragment length polymorphism technique, as described in Table 1.

The PCR reaction was done in a 20-μL reaction volume. The mixture contained 25 ng DNA TopTaq PCR Master Mix (Qiagen, Germany) with 0.01% bovine serum albumin as well as 10 μM primers for IL-10, 12.5 μM primers for IL-4, and 10 μM primers for CTLA-4. The reaction was performed using 2720 Thermal Cycler 96-well (Applied Biosystems, Life Technologies, Carlsbad, California). The annealing step conditions for each PCR reaction are mentioned in Table 1.

The PCR-restriction fragment length polymorphism assays for single-nucleotide polymorphism (SNP) genotyping were performed by treating the PCR products with restriction endonucleases (Thermo Fisher, Waltham, Massachusetts; Table 2) for 16 hours at 37°C. Analysis of the restriction fragments was done by electrophoresis on 6% (for *IL10*, *DAO,* and *CTLA4*) and 12% (for *IL4*) polyacrylamide gels before silver staining.

Table 1. Primers Used in the Polymorphism Genotyping of Cytokines, Diamino Oxidase, and Regulatory Molecule CTLA-4 and Annealing Temperatures Used in the Polymerase Chain Reaction

SNP Position	Primers	Reference	Annealing Temperature
IL-10	F: 5'-CCAAGACAACACTACTAAGGCTCCTTT-3'	Oliveira et al[34]	56°C, 35 s
(–1082 G>A)	R: 5'-GCTTCTTATATGCTAGTCAGGTA-3'		
IL-4	F: 5′-ACTAGGCCTCACCTGATACG-3′	Oliveira et al[34]	59°C, 1 min
(-589C>T)	R: 5′-GTTGTAATGCAGTCCTCCTG-3′		
CTLA-4	F: 5′-AAGGCTCAGCTGAACCTGGT-3'	Rodrigues et al[34]	58°C, 30 s
(+49 A>G)	R: 5′-CTGCTGAAACAAATGAAACCC-3′		
DAO	F: 5′-GGTCACCTGAACCCGGTTAAC-3′	Ayuso et al[15]	61° C, 1 min
(+8956 C>G)	F: 5′- TTGTGACCTCTGAACTTGCCG-3′		

CTLA indicates cytotoxic T lymphocyte antigen; DAO, diamino oxidase; F, forward; IL, interleukin; R, reverse; SNP, single nucleotide polymorphism.

Table 2. Restriction Fragment Length Polymorphism Technique: Polymorphism Position, PCR Products, Restriction Enzymes, and Size of the Restriction Fragments

Polymorphism Position	PCR Products (Base Pairs)	Restriction Enzyme	Digestion Fragments (Base Pairs)
IL-10 (-1082 G>A)	377	*XagI*	280 + 97 (allele A) 253+97+27 (allele G)
IL-4 (-589C>T)	195	*Ava II*	195 (allele T) 174 + 21 (allele C)
CTLA-4 (+49 A>G)	152	*BstEII*	152 (allele G) 130 + 22 (allele A)
DAO (+8956 C>G)	476	*Ava II*	369 + 107 (allele C) 252 + 117 + 107 (allele G)

CTLA indicates cytotoxic T lymphocyte antigen; DAO, D-amino acid oxidase; IL, interleukin; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Statistical Analysis

Comparisons between cases and controls were performed using unconditional logistic regression models controlling for the potential confounders sex and age (continuous). All SNPs were analyzed under dominant and codominant models. A stepwise logistic regression analysis was performed to select the SNPs and covariates for a multiple model. Interactions between SNPs were assessed by including interaction terms in logistic regression models. The Cochran-Armitage trend test was used to determine possible allele dose effects. All analyses were performed using R for windows, version 2.14.1, with the packages "genetics" and "coin."

Additionally, Hardy-Weinberg equilibrium was tested for each SNP using Michael H. Court's (2005-2008) online calculator.[20] The odds ratio (OR) with a confidence interval of 95% was calculated in order to evaluate the risk associated with genotypes (individual or combined). We considered a value of $P \leq .05$ to be statistically significant.

Results

Frequency of SNPs at *IL10*, *IL4*, *CTLA4,* and *DAO* Genes in Cases and Controls and Association With NSAID Hypersensitivity

Genotype distribution and allele frequencies of *IL10* –1082 A>G, *IL4* –589 C>T, *CTLA4* +49 A>G, and *DAO* +8956 C>G in cases and controls are shown in Table 3. No deviations from Hardy-Weinberg equilibrium were observed in the control group. Results of logistic regression models showed an association between *IL10* –1082 A>G and increased risk of hypersensitivity reaction (Table 3), with OR values of 2.57 for AG and 2.38 for GG carriers ($P = .03$ for the codominant model). Accordingly, analysis under a dominant model showed increased risk of hypersensitivity reaction for allele G carriers (AG/GG; OR = 2.52; $P = .01$).

By contrast, the SNP *DAO* +8956 C>G was associated with a strong protective effect, with OR values of 0.83 for CG and 0.11 for GG genotype ($P = .04$ for the codominant model), suggesting an allele dose effect (Table 3). The frequency of heterozygotes was similar in cases (0.50) and controls (0.48), whereas GG homozygotes showed a very low frequency among cases (0.02) as compared to controls (0.13). Accordingly, results of the dominant model were not statistically significant, suggesting that the effect may be specific for homozygotes. However, this trend was not supported by the Cochran-Armitage trend test ($P = .07$). Similar results were obtained for *CTLA4* +49 A>G SNP, with OR values suggesting a protective effect for AG genotype (OR = 0.41; $P = .02$) and G carriers (AG/GG; OR = 0.51; $P = .05$).

The frequency of *IL4* –589 C/T was also increased among controls, suggesting a protective effect. However, no significant results were observed (Table 3). Results obtained for all SNPs remained virtually the same after adjustment for age and sex (Table 3).

Gene-Gene Interaction and Multivariate Analysis

Pairwise gene-gene interaction analyses were performed by including interaction terms in logistic

Table 3. Association Between Candidate SNPs at *IL10*, *IL4*, *CTLA4,* and *DAO* Genes and Hypersensitivity to NSAIDs

		N (Frequency)		OR [95%CI; *P*]	
SNP	Genotype	Cases	Controls	Crude	Adjusted[a]
IL10	AA	16 (0.30)	49 (0.52)	Reference	Reference
	AG	31 (0.57)	37 (0.39)	2.56 [1.22-5.37; $P = .01$]	2.62 [1.22-5.59; $P = .01$]
	GG	7 (0.13)	9 (0.09)	2.38 [0.76-7.42; $P = .13$]	2.47 [0.77-7.89; $P = .12$]
		54	95	$P = .03$	$P = .04$
	AG/GG	38 (0.70)	46 (0.48)	2.52 [1.24-5.14; $P = .01$]	2.59 [1.25-5.38; $P = .01$]
IL4	CC	28 (0.52)	35 (0.37)	Reference	Reference
	CT	21 (0.39)	45 (0.47)	0.58 [0.28-1.19; $P = .14$]	0.56 [0.27-1.15; $P = .11$]
	TT	5 (0.09)	15 (0.16)	0.41 [0.13-1.28; $P = .12$]	0.41 [0.13-1.28; $P = .12$]
		54	95	$P = .17$	$P = .15$
	CT/TT	26 (0.48)	60 (0.63)	0.54 [0.27-1.06; $P = .08$]	0.52 [0.26-1.03; $P = .06$]
CTLA4	AA	25 (0.46)	29 (0.31)	Reference	Reference
	AG	17 (0.31)	48 (0.51)	0.41 [0.19-0.88; $P = .02$]	0.41 [0.19-0.89; $P = .02$]
	GG	12 (0.22)	18 (0.19)	0.77 [0.31-1.91; $P = .57$]	0.74 [0.30-1.86; $P = .53$]
		54	95	$P = 0.06$	$P = 0.07$
	AG/GG	29 (0.54)	66 (0.69)	.51 [0.25-1.01; $P = .05$]	.50 [0.25-1.01; $P = .05$]
DAO	CC	26 (0.48)	37 (0.39)	Reference	Reference
	CG	27 (0.50)	46 (0.48)	0.83 [0.42-1.66; $P = .60$]	0.83 [0.41-1.66; $P = .59$]
	GG	1 (0.02)	12 (0.13)	0.11 [0.01-0.97; $P = .04$]	0.12 [0.01-0.97; $P = .04$]
		54	95	$P = .04$	$P = .04$
	CG/GG	28 (0.52)	58 (0.61)	0.69 [0.35-1.35; $P = .27$]	0.68 [0.34-1.35; $P = .27$]

NSAID indicates nonsteroidal anti-inflammatory drug; OR, odds ratio; SNP, single nucleotide polymorphism.
[a]Results were adjusted for age and sex.

Table 4. Multivariate Analysis

Gene	Genotype	OR [95%CI]	*P*-Value[a]
IL10	AA	Reference	.03
	AG	2.61 [1.22-5.55]	
	GG	2.38 [0.75-7.58]	
DAO	CC	Reference	.04
	CG	0.86 [0.42-1.76]	
	GG	0.12 [0.01-0.96]	

OR indicates odds ratio.
[a]$P = .03$ for comparisons between a model including only *IL10* and the multiple model.

regression models. Statistically significant interactions were not observed for any pair (data not shown). Finally, a stepwise logistic regression analysis was performed to define the best model to explain NSAIDs hypersensitivity considering the genetic and nongenetic variables investigated in the study. Results of multivariate logistic regression models including *IL10* –1082 A>G and *DAO* +8956 C>G SNPs are shown in Table 4. The OR values obtained for *IL10* and *DAO* genotypes were similar to those observed in univariate models. Taken together, these results suggest that *IL10* and *DAO* SNPs exert independent effects in susceptibility to NSAID hypersensitivity.

Discussion

In the last decade the most frequent studies related to genetic factors affecting hypersensitivity to NSAIDs have included leukotriene synthesis and drug metabolism enzymes such as cytochrome P450 isoenzymes, diamino oxidase, and HLA genes.[16] Although it is thought that leukotriene overproduction is the main nonallergic mechanism found in hypersensitivity to NSAIDs, there are some events that occur in nonallergic hypersensitivity to NSAIDs cases that are not explained by this type of mechanism. For instance, antihistamine administration is necessary for therapeutic management, although the mechanism of histamine secretion is unknown. After its release, histamine may undergo oxidative deamination catalyzed by the diamino oxidase enzyme to imidazole acetaldehyde, which is converted to an imidazoleacetic acid riboside and excreted in urine.[21] The gene encoding the diamino oxidase enzyme is located on chromosome 7, q34-q36. Nonsynonymous SNPs were found to affect the enzyme activity.[15] In the present study, we

evaluated the *DAO* +8956 C>G polymorphism, which causes a substitution on His645Asp,[15], and we verified that those who were homozygous to GG presented lower risk of having hypersensitivity to NSAIDs ($P = .04$, OR = 0.11, 95%CI = 0.01-0.97). Agundez et al[22] found increased frequency of the polymorphic variant in diamino oxidase gene 16 Met allele (rs10156191) and lower ability to metabolize histamine in subjects with hypersensitivity to NSAIDs.

Another intriguing point is the high frequency of certain major histocompatibility complex alleles among patients with cross-reactive hypersensitivity to NSAIDs. Quiralte et al[23] found a higher frequency of HLA-DR11 alleles in cases compared to controls. Some hypotheses are proposed for the role of major histocompatibility complex molecules in drug allergy but not in nonallergic hypersensitivity.[24]

Kurosawa et al[25] demonstrated that the frequency of TT/CT genotypes in IL-13 polymorphism –1111 was higher than that of the CC genotype in NSAID-exacerbated asthma disease compared to an aspirin-tolerant group and controls. Interleukin-13 is a cytokine derived from Th2 cells that induces IgE production by B cells and retains a main role in allergic-induced asthma. According to Cameron et al,[26] the –1112 T allele drives IL-13 transcription in the Th2 milieu. Regarding this subject, we decided to analyze the polymorphism of IL-10, IL-4 cytokines, and the CTLA-4 molecule in nonallergic hypersensitivity to NSAIDs.

IL-10 is an important cytokine in the immunoregulation and control of inflammation because it inhibits the production of Th1 and Th2 cytokine profiles.[27] IL-10 is a key cytokine of the regulatory T profile produced by T and B lymphocytes and other cells. In the presence of IL-10, T lymphocytes do not proliferate and do not produce cytokines in response to an allergen. The IL-10 gene contains 5 exons and is located on the long arm of chromosome 1, between 1q31 and 1q32.[28] In the proximal promoter region of the IL-10 gene, 3 SNPs located at the –1082 (A>G), –819 (T>C), and –592 (C>A) positions can be found. The level of production of the cytokine is mainly determined by the synthesis rate of mRNA, which in turn depends on the polymorphism in the promoter.[29] Regarding the –1082 position of the IL-10 promoter region, studies have shown that the GG genotype drives a high production of this cytokine, the GA genotype an intermediate production of IL-10, and the AA genotype a low production of the cytokine.[30] With regard to the *IL10* –1082 polymorphism, the G allele carriers were found to be significantly associated with hypersensitivity to NSAIDs ($P = .01$). This would mean that high production of IL-10 would be a risk factor for nonallergic hypersensitivity (OR = 2.52; 1.24-5.14). We have observed in a previous report that the –1082 AA genotype was much more frequent in HIV-positive patients allergic to efavirenz than in nonallergic patients ($P = .019$; OR = 3.625; 95%CI = 1.210 to 10.860).[31] Comparing our present work with previous reports, one can hypothesize that low production of IL-10 is a risk factor for drug allergy but a protection against nonallergic hypersensitivity.

IL-4 is a cytokine that plays a central role in the development of allergic asthma and atopy. Rosenwasser et al[32] found that patients homozygous for the polymorphic T allele (–589 C/T) presented higher production of IL-4. Therefore, gene polymorphisms of this cytokine can alter its transcription and translation, influencing the pathogenesis of allergic diseases.[33] The *IL4* gene is located on chromosome 5q31, and to date, about 19 polymorphisms in this gene are known. We found a combined association of the IL-4 –589T allele (TT + CT) with the IL-10 –1082A allele (AG + AA) in allergy to efavirenz ($P = .015$; OR = 15.970, 95% CI = 0.8477-300.9),[34] that is, association between high IL-4 and low IL-10 production in efavirenz allergy. In the present study, we found no association between cases and controls for genotype and allele frequencies in IL4 –589 C>T. However, after adjustment for age and sex, it was observed that T carrier subjects (CT+TT) showed a tendency for lower risk of presenting adverse hypersensitivity to NSAIDs (OR = 0.52, 95%CI = 0.26-1.03; $P = .06$).

The CTLA-4 molecule is a member of the immunoglobulin superfamily expressed on activated T cells. It binds to the B7 molecule on the surface of antigen-presenting cells, resulting in negative signals to the T cell, affecting activation, proliferation, cytokine production, and immune response.[35,36] Polymorphisms in the *CTLA4* gene, especially in the +49 A>G coding region, affect immunoregulation and augment the risk of autoimmune and allergic diseases. The G allele is related to a lower expression of CTLA-4 molecules on the surface of T cells, interfering in downregulation of cellular activation. It was found that this polymorphism was associated with some autoimmune diseases such as Graves disease, thyroiditis of Hashimoto, multiple sclerosis, systemic lupus erythematosus,[37,38] asthma, and rhinitis as well.[39] Our results showed that, for *CTLA4* +49 SNP, G allele carriers (AG+GG) presented a lower risk of having hypersensitivity to NSAIDs (OR = 0.51, 95%CI = 0.25-1.01; $P = .05$).

As a way to detect possible interaction or confounding between the effects of the different SNPs, we have also performed gene-gene interaction and stepwise logistic regression analysis. A statistically significant model was obtained when *IL10* and *DAO* SNPs were combined. However, the OR values for each genotype were similar to those obtained in single-SNP

models, suggesting that the effects of these SNPs are independent.

In conclusion, these results suggest that polymorphisms in regulatory targets of the immune response and in *DAO* gene could modulate an individual's susceptibility to NSAID hypersensitivity reactions. Further studies will be necessary to complement our results.

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Contributions of Authors

All authors made substantial contributions to conception, analysis, and interpretation of data for this work.

Author Disclosure Statement

No competing financial interests exist.

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