The Pattern of Methylation and Polymorphism in Interleukin-6 Promoter Gene Are Related to the Development of Rheumatoid Arthritis?

Isaura Isabelle Fonseca Gomes-Silva[1], Eliezer Rushansky[2], Francisco Geraldo Carvalho-Neto[1], Amanda Virginia Batista Vieira[3], Maria Helena Queiroz de Araujo Mariano[2], Paulo Roberto Eleutério de Souza[3], Martín Alejandro Montes[3], Maria Mascena Diniz Maia[3]

[1] Department of Genetics, Federal University of Pernambuco, Av. Prof. Moraes Rego, 1235-Cidade Universitária, Recife-PE, Brazil

[2] Department of Clinical Rheumatology, University of Pernambuco, Rua Arnóbio Marques, 10-Santo Amaro, Recife-PE, Brazil

[3] Department of Biology, Rural Federal University of Pernambuco, Rua Manoel de Medeiros-Dois Irmãos, Recife-PE, Brazil

Received: 08 Apr. 2017; Accepted: 12 Dec. 2017

Abstract- Rheumatoid Arthritis (RA) is a complex disease with higher level of *IL-6*. In order to elucidate the alterations related to *IL-6* gene in RA, we evaluated the -174 G/C *IL-6* gene polymorphism and methylation pattern of its promoter. A total of 120 RA patients and 120 healthy controls were recruited for polymorphism analysis, and 30 individuals of both groups were randomly selected for methylation analysis. The *IL-6* gene polymorphism was analyzed by *PCR-RFLP* and methylation pattern was analyzed by *MSP-PCR*. Regarding *IL-6* gene polymorphism, no significant difference was found between RA patients and controls (P>0.05). For DNA, two CpG regions of *IL-6* promoter gene was analyzed. The first region (−1069, −1061, −1057 and −1001 *CpG*) did not show significant differences, whereas the second region (−628, −610, −574 and −491 *CpG*) showed a significant hypermethylated status (P=0.0004) in RA patients as compared with controls.These results, suggest a possible relationship between methylation pattern and the susceptibility to RA in our studied population.

Acta Med Iran 2018;56(7):429-433.

Keywords: Cytokine; Inflammation; Epigenetics; *MSP-PCR;* Single nucleotide polymorphism

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease of unknown etiology. Genetics immunological and epigenetic factors may contribute to RA susceptibility (1,2). Many cytokines are expressed in the process of this pathology (3), among them, the interleukin-6 (*IL-6*) that is a pleiotropic cytokine that can promote inflammation through to differentiation of T *helper* cells and proliferation of osteoclasts and synoviocytes (4,5). Besides this, has been documented higher levels of *IL-6* in serum and synovial fluid of RA patients (6). Studies have shown that high concentrations of cytokines may be related to genetic and epigenetic variations in RA subjects and consequently affected on susceptibility and severity of the disease (1,2). Besides that, studies involving polymorphism in cytokines genes have been associated with RA susceptibility (7,8). The -174 G/C *IL-6* (rs1800795) gene polymorphism, is responsible for altering levels of cytokine in vivo (9) and was associated with inflammatory diseases, as like RA (7), acute rheumatic fever (10) and osteoarthritis (11). DNA methylation is an epigenetic modification that affects gene silencing and occurs mainly in CpG-rich islands (1). Recent studies have reported that methylation of *CpG* motifs is a potential mechanism of regulation of *IL-6* production that may be relevant to the pathogenesis of inflammatory diseases (12,13). Based on this information we hypothesized that the -174 G/C (rs1800795) *IL-6* gene polymorphism and *IL-6* gene promoter methylation pattern might constitute to both epigenetic and genetic susceptibility factors for RA in patients from Pernambuco, Brazil**.**

Materials and Methods

Study population

In our present study, 120 RA patients (cases) and

Corresponding Author: M. Mascena Diniz Maia
Department of Biology, Rural Federal University of Pernambuco, Rua Manoel de Medeiros-Dois Irmãos, Recife-PE, Brazil
Tel: +55 81 999691052 Fax: +55 81 33206300, E-mail address: mascenadiniz@hotmail.com

120 healthy individuals (controls) were selected from the investigation of Division of Clinical Rheumatology of the Hospital Oswaldo Cruz-Recife, Pernambuco, Brazil. The patients were diagnosed according to American College of Rheumatology/European League Against Rheumatism classification criteria (14). Blood samples were collected for DNA analysis. Control subjects consisted of healthy volunteers between 20 and 80-year-old with the same study to match RA in terms of age, gender, smoking status and proportion that patients group. The individuals were included in control group if their medical history did not reveal RA, other autoimmune or infectious disease. If some of these conditions were showed in any individual, we excluded from the control group. The methylation analysis were performed using 30 RA patients and 30 healthy control subjects selected randomly. All subjects inhabited the same geographic area and were recruited between July 2015 and May 2016. The study was approved by the Ethics Committee of the Health Sciences Center of Federal University of Pernambuco (protocol 1.097.918/2015). Written informed consent was obtained from all participants.

DNA isolation

Genomic DNA was isolated from peripheral blood leukocytes using Wizard Genomic DNA Purification Kit (PROMEGA, Madison, USA) according to the manufacturer's descriptions. The isolated DNA was stored at -20° C.

Bisulfite conversion and methylation-specific polymerase chain reaction (MSP)

Five hundred nanograms of genomic DNA were treated with bisulfite follows the Pappas *et al*., (15) instructions, to modify unmethylated cytokines to uracils, whereas methylated cytokines must remain unaltered. Bisulfite-treated DNA was purified and resuspended in 50 µl of TE (10 mM Tris -1 mM EDTA) buffer pH 8.0. Amplification of the bisulfite-treated DNA was performed based on Stefani *et al*., (13), to assess two regions of the *IL-6* promoter gene containing eight *CpG* motifs (MSP1: −1069, −1061, −1057 and −1001 and MSP2: −628, −610, −574 and −491) (Genbank Accession No.: AY170325-1). All amplification products were separated by electrophoresis on 2% agarose gel. It was performed two independent experiments to ensure the reproducibility of the technique.

Genotyping

The -174 G/C *IL-6* gene polymorphism, was detected by PCR followed by restriction enzyme fragment analysis (*PCR-RFLP*). The primers used were as follows: forward 5'-TTGTCAAGACATGCCAAAGTG-3' and reverse 5'-CTGATTGGAAACCTTATTAGG-3'. The products were digested with *Hsp92II* restriction enzyme, as previously described (16). The PCRs consisted of a pre-denaturation step of 4 min at 94° C and 40 cycles each of 30 s denaturation at 94° C, 30 s annealing at 55° C and 30 s elongation at 72° C. This was followed by a post-elongation step of 7 min at 72° C. Restriction endonuclease was obtained from Fermentas (st. Leon-rot, Germany) and was used as described by the manufacturer. Restriction fragments were visualized by electrophoresis on 3% agarose.

Statistical analysis

A descriptive statistical approach was used initially with a data set created from information such as genotyping and methylation patterns. Univariate analysis was performed to evaluating the association between RA development and genotyping. In addition, we performed a logistic regression with adjustment for age and gender to assess the association between development of RA and methylation patterns. Differences in genotype distribution and allele frequencies, as well as features in all subjects, were evaluated using the chi-square test with 95% confidence intervals (CIs), and the p-value was considered significant below 0.05. The genotype distributions were assessed by the Hardy-Weinberg equilibrium proportion tests. All analyzes were performed in R version 3.2.1 (17). The post-hoc statistical power calculation was performed using G Power 3.1 software.

Results

Demographics and risk factors of all patients were described (Table 1). Mean age of patients with RA was 52.86±10.33 years is 97.5% women. For the control group, the mean age was 48.79±12.47 years being 95% women. The RA patients selected for methylation analysis had mean age 54.46±9.85 years, of these, 96.67% women, whereas controls groups had mean age 55.44±7.95 years as 93.3% women.

Table 1. Clinical features of the patients with rheumatoid arthritis (RA)

Variable	RA patients (*N*=120)	RA patients methylation analysis (N=30)
Gender Female/ Male; *n* (%)	117(97.5) / 3(2.5)	29 (96.67) / 1 (3.33)
Age; mean (range) years	52.86 ± 10.33	54.46 ± 9.85
Age at RA onset; mean (range) years	41.86 ± 10.18	43.67 ± 9.94
Disease duration, mean (range) years	11.00 ± 7.88	10.8 ± 8.14
CRP, means (range) mg/L	5.08 ± 10.43	7.98 ± 12.98
ESR; mean (range) mm/h	22.49 ± 12.8	21.9 ± 12.27
Rheumatoid factor positive, *n* (%)	53 (44.16)	11 (36.67)
ACPA present[a], *n* (%)	15 (75.00)	--
DAS28 Low (2.6 < DAS28 ≤ 3.2)	13 (10.83)	1 (3.33)
Moderate (3.2 < DAS28 ≤ 5.1)	87 (72.5)	22 (73.33)
Severe (> 5.1)	6 (5.0)	3 (10.0)
HAQ; mean (range)	1.43 ±0.66	1.33 ± 0.73
CDAI; mean (range)	20.33 ± 10.02	20.5 ± 11.62
Erosions present, *n* (%)	102 (85.0)	11 (36.67)
Smoke; *n* (%)	15 (12.5)	4 (13.33)
Treatment with biological DMARDs *n* (%)	59 (49.17)	11 (36.67)

ESR: Erythrocyte sedimentation rate; ACPA: Anti-cyclic citrullinated peptide antibodies; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; DAS28: RA disease activity score; HAQ: Health Assessment Questionnaire and CDAI: Clinical Disease Activity Index.
[a]- Data available for 20 patients

The genotype and allele frequencies of the *IL-6* promoter gene in RA are shown (Table 2). No association between genotype distribution and RA was detected (*P*=0.694), the same was observed in relation to allele frequency (*P*≅1.00). The methylation pattern of the *IL-6* promoter gene also was described (Table 2). The *CpG* motifs MSP1 (−1069, −1061, −1057 and −1001) in RA and healthy groups were predominantly partially methylated, besides in the logistic regression model adjusted for age and gender, no significant differences were observed between levels of methylation of RA and healthy groups (*P*>0.05). The *CpG* motifs at MSP2 (−628, −610, −574 and −491) was significantly most methylated in RA as compared with healthy controls (80.0% versus 33.3%; *P*=0.0004 by chi-square test).

Table 2. Genotype distribution and allele frequency of -174 G/C *IL-6* (rs1800795) gene polymorphism and methylation pattern of the *IL-6* promoter gene in Rheumatoid Arthritis patients and healthy controls

-174 G/C *IL-6* polymorphism		RA patients N=120 N (%)	Controls N=120 N (%)	*P**	OR (95% CI)	*P*
Genotype[a]	**GG**	68 (56.66)	72 (60.0)		Reference	
	GC	50 (41.66)	43 (35.83)			
	CC	2 (1.66)	5 (4.17)	0.381	0.87 (0.52-1.45)	0.694
Allele [a]	**G**	186 (77.5)	187 (77.92)			
	C	54 (22.5)	53 (22.08)		0.97 (0.63-1.50)	1.000
Methylation Pattern		**RA patients N=30 N (%)**	**Controls N=30 N (%)**	***P****	**OR (95% CI)**	***P***
MSP1[b]	**MM**	2 (6.67)	3 (10.0)		Reference	
	UM	15 (50.0)	17 (56.67)		0.96 (0.29-27.3)	0.381
	UU	13 (43.33)	10 (33.33)	0.699	1.34 (0.38-44.5)	0.248
***MSP2* [b]**	**MM**	24 (80.0)	10 (33.33)		Reference	
	UM	4 (13.33)	19 (63.34)		0.37 (0.15-0.25)	<0.001
	UU	2 (6.67)	1 (3.33)	0.0004	0.16 (0.08-34.5)	0.907

Bold means significance; P* - P value of χ^2; OR – odds ratio; CI – confidence interval; P - P value of OR; MM - total methylation; UM - partial methylation; UU – unmethylation; [a]_ Univariate Analyses; [b]- Logistic regression analyses, adjusted for age and gender

Discussion

In relation to -174 G/C *IL-6* gene polymorphism, no significant difference between RA patients and healthy controls was observed. Even though, the power of our study is not high due to the sample size (Power (1–β err prob)=0.50), similar outcomes were found in Mexican (18), Spanish (19) and Turkish (20) populations. However, literature data show contradictory results since another study in Chinese (7) and Egyptian (21) populations indicates association of the polymorphism with the risk for RA development. Indeed, a meta-analysis suggests that this polymorphism has been associated with RA risk in Asian, but not in Caucasian populations (22). Although this polymorphism has been related to altering levels of cytokines in vivo assays (6,9), there is no consensus whether RA is associated or not with this polymorphism, since lack association found in many studies can be explained due to the low frequency of C variant in different ethnics groups.

We analyze the methylation pattern in two different regions of the *IL-6* gene (MSP1 and MSP2). Logistic regression analyses did not reveal difference in first region MSP1 (−1069, −1061, −1057 and −1001 *CpG* motifs). Similar results were found by Nile *et al.*, (12) and Ishida *et al.*, (23) in RA and Stefani *et al.*, (13) studying periodontitis also showed similar methylation pattern between patients and controls. Nevertheless, for the second region MSP2 (−628, −610, −574 and −491 *CpG* motifs) RA patients showed higher level of methylation as compared with healthy controls. Contradictory results were found by Nile *et al.*, (12) and Ishida *et al*., (23), which did not verify hypermethylation in promoter *IL-6* gene. Besides this, other study with RA patients and *TNF-α* gene indicted hypermethylation of *CpG* motifs in patients group as compared with controls (24). In other diseases as obesity which involve inflammatory process, it were found a hypermethylation pattern of *IL-6* gene most frequent in patients than controls group (25). These findings can be explained by different hypothesis: the persistent inflammation could cause global DNA hypermethylation of pro-inflammatory cytokines or the specific characteristics of our study population as ethnic, environmental exposures, disease duration, disease activity or laboratory features of the patients may influence the methylation pattern. Moreover, Ishida *et al*., (23) suggested that hypermethylation may be a result of increased DNA methyltransferases activity as a way to decrease the inflammatory process.

In conclusion, the -174 G/C *IL-6* gene polymorphism did not play an important role in RA development in our population even though the hypermethylated status of *CpG* motifs in the *IL-6* promoter region may implicating a role in RA in the population of Pernambuco, Brazil.

Acknowledgements

We thank the patients who generously agreed to participate in this study. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Trenkmann M, Brock M, Ospelt C, Gay S. Epigenetics in Rheumatoid Arthritis. Clin. Rev. Allergy Immunol 2010; 39:10-9 .
2. Trajkov D, Mishevska-Perchinkova S, Karadzova-Stojanoska A, Petlichkovski A, Strezova A, Spiroski M. Association of 22 cytokine gene polymorphisms with rheumatoid arthritis in population of ethnic Macedonians. Clin Rheumatol 2009;28:1291-300 .
3. Gierut A, Perlman H, Pope RM. Innate Immunity and Rheumatoid Arthritis. Rheum. Dis Clin N Am 2010;36:271-96 .
4. Yoshida Y, Tanaka T. Interleukin 6 and Rheumatoid Arthritis. Biomed Res Int 2014;2014:698313 .
5. Le Goff B, Blanchard F, Berthelot JM, Heymann D, Maugars Y. Role for interleukin-6 in structural joint damage and systemic bone loss in rheumatoid arthritis. Joint Bone Spine 2010;77:201-5 .
6. Solus JF, Chung CP, Oeser A, Li C, Rho YH, Bradley KM, et al. Genetics of serum concentration of IL-6 and TNFα in systemic lupus erythematosus and rheumatoid arthritis: a candidate gene analysis. Clin Rheumatol 2015;34:1375-82 .
7. Li X, Chai W, Ni M, Xu M, Lian Z, Shi L, et al. The Effects of Gene Polymorphisms in Interleukin-4 and Interleukin-6 on the Susceptibility of Rheumatoid Arthritis in a Chinese Population. Bio Med Res 2014;2014:265435 .
8. Angelo HD, Gomes Silva IIF, Oliveira RDR, Louzada-Júnior P, Donadi EA, Crovella S, et al. Interleukin-18, interleukin-12B and interferon-γ gene polymorphisms in Brazilian patients with rheumatoid arthritis: a pilot study: IL-18, IL-12B and IFN-γ gene polymorphisms and rheumatoid arthritis. Tissue Antigens 2015;86:276-8 .
9. Bennermo M. Genetic Predisposition of the Interleukin-6

Response to Inflammation: Implications for a Variety of Major Diseases? Clin Chem 2004;50:2136-40 .

10. Col-Araz N, Pehlivan S, Baspinar O, Oguzkan-Balci S, Sever T, Balat A. Role of cytokine gene (IFN-γ, TNF-α, TGF-β1, IL-6, and IL-10) polymorphisms in pathogenesis of acute rheumatic fever in Turkish children. Eur J Pediatr 2012;171:1103-8 .
11. Pola E, Papaleo P, Pola R, Gaetani E, Tamburelli FC, Aulisa L, et al. Interleukin-6 gene polymorphism and risk of osteoarthritis of the hip: a case–control study. Osteoarthritis Cartilage 2005;13:1025-8 .
12. Nile CJ, Read RC, Akil M, Duff GW, Wilson AG. Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum 2008;58:2686-93 .
13. Stefani FA, Viana MB, Dupim AC, Brito JA, Gomez RS, da Costa JE, et al. Expression, polymorphism and methylation pattern of interleukin-6 in periodontal tissues. Immunobiology 2013;218:10121-7 .
14. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24 .
15. Pappas JJ, Toulouse A, Bradley WEC. The bisulfite genomic sequencing protocol. Adv Lung Cancer 2013;2:21-5 .
16. Asano NM, Angelo HD, da Silva HA, Maia MM, Lins OG, Souza PE. Interleukin-6 promoter polymorphisms −174 G/C in Brazilian patients with systemic lupus erythematosus. Hum Immunol 2013;74:1153-6 .
17. Team RC, R. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2013. (Accessed March 4, 2017, at http.www.R-project.org.(
18. Zavaleta-Muñiz SA, Martín-Márquez BT, Gonzalez-Lopez L, Gonzalez-Montoya NG, Díaz-Toscano ML, Ponce-Guarneros JM, et al. Patients The −174G/C and −572G/C Interleukin 6 Promoter Gene Polymorphisms in Mexican with Rheumatoid Arthritis: A Case-Control Study. Clin Dev Immunol 2013;2013: 959084 .
19. Palomino-Morales R, Gonzalez-Juanatey C, Vazquez-Rodriguez TR, Miranda-Filloy JA, Llorca J, Martin J, et al. Interleukin-6 gene-174 promoter polymorphism is associated with endothelial dysfunction but not with disease susceptibility in patients with rheumatoid arthritis. Clin Exp Rheumatol 2009;27:964-70 .
20. Arman A, Coker A, Sarioz O, Inanc N, Direskeneli H. Lack of association between IL-6 gene polymorphisms and rheumatoid arthritis in Turkish population. Rheumatol Int 2012;32:2199-201 .
21. Amr K, El-Awady R, Raslan H. Assessment of the −174G/C (rs1800795) and −572G/C (rs1800796) Interleukin 6 Gene Polymorphisms in Egyptian Patients with Rheumatoid Arthritis. Open Access Maced J Med Sci 2016;4:574-7.
22. Dar SA, Haque S, Mandal RK, Singh T, Wahid M, Jawed A, et al. Interleukin-6-174G/C (rs1800795) polymorphism distribution and its association with rheumatoid arthritis: A case-control study and meta-analysis. Autoimmunity 2016;50:158-69 .
23. Ishida K, Kobayashi T, Ito S, Komatsu Y, Yokoyama T, Okada M, et al. Interleukin-6 Gene Promoter Methylation in Rheumatoid Arthritis and Chronic Periodontitis. J Periodontol 2012;83:917-25 .
24. Kojima A, Kobayashi T, Ito S, Murasawa A, Nakazono K, Yoshie H. Tumor necrosis factor-alpha gene promoter methylation in Japanese adults with chronic periodontitis and rheumatoid arthritis. J Periodontal Res 2016;51:350-8 .
25. Na YK, Hong HS, Lee WK, Kim YH, Kim DS. Increased Methylation of Interleukin 6 Gene Is Associated with Obesity in Korean Women. Mol Cells 2015;38:452-6.