THE PRESENCE OF PERIODONTAL PATHOGENS AND THEIR RELATIONSHIP WITH THE MBL2 GENE IN PATIENTS WITH CHRONIC PERIODONTITIS

Renata Cimões[1], Paulo Roberto Eleutério de Souza[2], Bruna de Carvalho Farias Vajgel[1], Rayanne Soraia Aguiar de Melo Dias[3], Betânia Ferreira[3]

ABSTRACT:

Objective: Considering the role of MBL2 in innate immunity and its importance for periodontitis — infectious multifactorial disease —, this study aimed to evaluate the association of the MBL2 gene polymorphism with the presence of periodontal pathogens in patients with chronic periodontitis. **Methods:** The convenience sample consisted of 29 patients diagnosed with chronic periodontitis who sought dental treatment from August 2008 to July 2009. Samples were collected from subgingival plaque of the four sites with deepest probing depth and the presence of periodontal pathogens was analyzed by PCR. Desquamated cells from oral mucosa were also collected to evaluate the MBL2 polymorphism by Q-PCR technique. **Results:** Regarding the MBL2, 19 (65.5%) patients presented the genotype A/A, 10 (34.5%) had genotype A/O, and none had genotype O/O. It was not observed significant association between the presence of periodontal pathogens and found genotypes ($p > 0.05$). **Conclusion:** Our results suggest that there is no association between genotypes and the presence of periodontal pathogens in patients with chronic periodontitis.

KEYWORDS:

Polymorphism, single nucleotide. Polymerase chain reaction. Chronic periodontitis.

1. Universidade Federal de Pernambuco, Centro de Ciências da Saúde, Curso de Pós-graduação em Odontologia (Recife/PE, Brazil).

2. Universidade Federal Rural de Pernambuco, Departamento de Biologia (Recife/PE, Brazil).

3. Universidade Federal de Pernambuco, Centro de Ciências da Saúde, Curso de Graduação em Odontologia (Recife/PE, Brazil).

DOI: https://doi.org/10.14436/2447-911x.15.2.170-179.oar

INTRODUCTION

The immune system is responsible for the organism's defense mechanism and it can be divided in two different types: the innate immune system, also known as natural immunity, which consists of the first line of defense against a great diversity of microorganisms. And the adaptive immune system or acquired immunity. Among the components present in the innate immunity there's the mannose binding-protein, or MBL[1]. The MBL is a protein that belongs to the collectin family, it's produced in the liver and circles in the bloodstream[2].

There are three activation paths to the complementary system: the classic path, which depends on the former generation of antibodies from the antibody-antigen complex, and the MBL path that takes part in the innate response, it's activation depends on specific components present in the surface of microorganisms[2]. When the MBL binds to the bacteria, it interacts with serum proteases originated from the blood plasma, forming a complex known as MBL associated to serum proteases, or MASPs. These, on the other hand, interact with C4 and activate the complements classic path, despite of the presence of antibodies[3].

The genes for human collectin genes are found in the chromosome 10(q21-24)[3]. There are two different MBL genes in humans, however the MBL1 is a pseudogene and only the MBL2 has the capacity of coding a protein as a final product[2]. The human MBL2 gene is placed in the q11.2-q21 arm, which codes the protein product called MBL2[3]. Its polymorphic sites are mostly referred to as alleles B, C and D. The allele A is considered normal, and the variant alleles are called O[2]. The homozygous subjects (0/0, where 0 can be B, C or D) for one of the mutant alleles produce quantities of MBL that can't be detected by ELISA (Enzyme-linked immunosorbent assay), meanwhile the heterozygous (A/0) have serum concentrations significantly lower when compared to homozygous subjects (A/A)[3].

Variations in DNA sequence that affect only one base are called single nucleotide polymorphism (SNPs) and work as molecular marker capable of distinguishing subjects through such variation[1], representing the most common genetic variation among subjects[4]. Therefore, the SNPs are considered a powerful weapon on identifying gens that are involved in multifarious, polygenic and

Mendelian diseases. The analysis of this type of mutation has been successful used in the better understanding of the pathogenesis of various diseases[2,4].

The MBL deficiency is relatively common in human populations and there are increasing evidences that it performs a complex role in various diseases[5]. Since then, the decrease of serum levels of such protein has been associated with multiple clinical alterations, such as recurring infections, especially in children; primary and secondary immunodeficiency, like infection by HIV; arteriosclerosis; cystic fibrosis; autoimmune diseases, such as lupus erythematosus; rheumatoid arthritis; heart diseases; diabetes mellitus[2]; periodic lung infections; recurrent otitis media; diarrhea and septicemia[6].

The periodontal diseases are multifarious infections caused by a range of bacteria species which interact with the hosts tissues and cells, leading to the release of mediators and substances which results on the destruction of periodontal structures when not treated[7]. Chronic periodontitis is the most frequent of all periodontitis[8], being clinically characterized by gum inflammation, bleeding during probing, decrease of periodontal tissue resistance to probing (periodontal pockets), and loss of insertion[9].

Bacteria are necessary, but only its presence is not enough for developing periodontal disease; the hosts immune response, genetic and environmental factors also partly determine the susceptibility and severity of this disease[2,6]. The development of periodontal disease seems to be determined by the relation between the bacterial plaque's periodontopathogenic potential and the hosts[2] immune response, therefore, the immune system plays an important role in the diseases pathogenesis and it is possible that the variations on the serum levels of MBL as a result of genetic polymorphism may take part in the periodontal disease susceptibility[2].

It is important to emphasize that periodontopathogens such as *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) present polysaccharides rich in mannose on its cell surface[2,6]. Therefore, MBL may play a fundamental role in the hosts defense against periodontal pathogens[2].

Once observed the relevance of the innate immune system in the organism's first line of defense against infections, this paper had as a goal to verify if there is any association between polymorphism of the MBL2 gene in patients who have chronic periodontitis and the presence of periodontopathogens, using techniques of molecular biology PCR (Polymerase Chain Reaction) and Q-PCR (Real-time Polymerase Chain Reaction), in a population of the city Recife, state of Pernambuco, in the northeast of Brazil.

METHODOLOGY

Participants and clinical exam

The current experimental and quantitative study has been approved by the Comity of Ethics in Research (CEP) under the process nº 191/2008. A convenient sample of 29 patients from both

sexes, diagnosed with chronic periodontitis who sought dental treatment in the period of August 2008 to July 2009, have participated in this study.

To take part in this research, the following criteria have been observed: to have at least 30 years old, present a minimum of 20 natural teeth, clinical and radiographic diagnostic of chronic periodontitis,[10] and to be willing to take part in the study. Were exclude from the study patients with the following conditions: smokers, patients with any systemic condition that may interfere in the diseases course (diabetes, hypertension, autoimmune diseases), pregnant or lactating women, patients who have been under periodontal treatment in the last 6 months, patients who have made use of antibiotics in the last 6 months, patients who made use of antiinflammatory drugs in a chronic way, HIV carriers and patients that used orthodontic braces.

After explaining the goals of the study, the patients were asked to sign a free and informed consent form. The oral exam was performed by a single calibrated examiner, in a clinic environment with artificial light and using tools such as oral mirror and periodontal Williams millimeter probe (Trinity®, São Paulo, Brazil).

The diagnosis of chronic periodontitis was made based on the criteria determined by AAP[11], according to which, to be considered a chronic periodontitis carrier the patient must present at least one site of probing depth and loss of clinical insertion ≥ 4 mm.

Sampling

From each patient, samples were collected from the plaque under the gum in the four sites with greater probing depth, for analyzing the presence of bacteria. Cones of #30 sterile paper (Dentsply, Petrópolis, Brazil) were inserted inside the pocket and remained there for 20 seconds, than were taken to Eppendorf-like tubes and stored at -20ºC, for later isolation of DNA and analysis by conventional PCR. It was also carried out the collection of cells from oral mucosa desquamation with Cytobrush brushes (Kolplast®, São Paulo, Brazil), which were later immersed in 1 ml of sodium chloride saline solution (Laboratory Tayuyna Ltda., Nova Odessa, Brasil). The collected material was stored at -20°C for later evaluation of the polymorphism of the MBL2 gene through Q-PCR.

DNA isolating

A commercial matrix of purification from the Geneclean kit (Qbiogene, Inc., CA, EUA) was used, following the protocol determined by the manufacturer.

Processing through conventional PCR technique for analysis of the pathogens

The amplification reaction was done with a total volume of 25µl containing 1,3µl of $MgCl_2$ at 50mM (LGC Biotecnologia, São Paulo Brazil), 2,5µl of dNTP 2mM (LGC Biotecnologia, Brasil), 1µl of each initializer and finisher at 10 µM (Invitrogen®, SP, Brasil), 2,5µl of 10x PCR buffer (LGC Biotecnologia, Brasil), 0,2µl of Taq DNA polymerase at 5U/µl (LGC Biotecnologia, Brasil), 13,5µl of injection water and 3µl of

DNA from the sample. In all amplification reactions, a negative control was used to verify the possibility of contamination.

The thermocycler (Peltier Thermal Cycler MJ96G, Biocycle Co. Ltd, China) was programmed according to Ávila-Campos, Velásquez-Meléndez study[12]. The primers used during the PCR in this study were species-specific for the 16S rDNA portion, and the sequence used was according to Ashimoto et al[13] (Table 1).

After this step, 9,5µl of the products from the PCR were added to a 0,5 µl of the Blue Green fluorescent dye (LGC Biotecnologia, Brasil) and subjected to electrophoresis in agarose gel at 1,5%. Later, the migrations of electrophoresis were observed under ultraviolet light and photographed for analysis. The standard molecular mass of 100 pb ladder (LGC Biotecnologia, Brasil) was included in the migrations.

Processing through PCR technique in real time for genotyping the MBL2 gene

The detection of polymorphism in the MBL2 gene was made using the Q-PCR technique and through the analysis of the melting temperature. The genotyping was done using the following primers drawn with the Primer Express 1.5 software (Applied Biosystems, CA, USA): primer "forward" 5'-AGGCATCAACGGCTTCCCA-3' and primer "reverse" 5'-CAGAA CAGCCCAACACGTACCT-3'. The amplification reactions were processed with a final volume of 25 µl, having 12,5 µl 1X SYBR Green (LGC Biotecnologia), 1,0 µl of primer "forward" 10Mm, 1,0 µl of primer "reverse" 10mM, and 3 µl of genomic DNA. The cycles presented the following conditions: initial heating of 95° C for 10 minutes, followed by 45 cycles with stages of 95° C for 30 minutes and 60° C for 1 minute. At the end of Q-PCR, a slow and gradual heating to 95° C was generated by increasing 0,2° C at every 8 seconds, to obtain the melting curves, using the dissociation software of the Rotor Gene Q (Qiagen Sample and Assay Technologies, Hilden, Germany)[14] as a platform.

The evaluation of the melting temperature helped to distinguish the profiles of the three different genotypes of the MBL2 gene (AA, A0 and 00): A/A (one peak of 83.1 ± 0.1ºC), A/0 (two peaks of 82.6 ± 0.3 e 80.7 ± 0.1ºC), and 0/0 (one peak of 81.7 ± 0.1ºC)[15].

The genotyping was done overlaying the melting curve of the patients on the melting curve of the three control samples: one wild, one mutant and one heterozygous. When there was an overlaying of those curves, it was possible to infer about the genotype in question.

Statistical analysis

In the analysis of the data, absolute distributions and univariate and bivariate percentages were obtained (descriptive statistics techniques) and the Fisher's exact test was used, since the conditions for using the Chi-squared test were not verified (inferential statistics techniques). The margin of error used for deciding the statistic tests was 5,0%.

Table 1:

Microorganisms and specific primers for conventional PCR.

MICROORGANISM	PRIMER	PCR ANNEALING TEMPERATURE	EXTENSION OF AMPLICON (BP*)
Aa	F‡ – GCT AAT ACC GCG TAG AGT CGG R‡ – ATT TCA CAC CTC ACT TAA AGG T	50^{0}C	557
Pg	F‡ – AGG CAG CTT GCC ATA CTG CG R‡ – ACT GTT AGC AAC TAC CGA TGT	60^{0}C	404
Tf	F‡ – GCG TAT GTA ACC TGC CCG CA R‡ – TGC TTC AGT GTC AGT TAT ACC T	60^{0}C	641
Td	F‡ – TAA TAC CGA ATG TGC TCA TTT ACA T R‡ – TCA AAG AAG CAT TCC CTC TTC TTC TTA	60^{0}C	316

**bp = base pairs. ‡ F = forward. Tf = Tannerella forsythia. ‡ R = reverse. Td = Treponema denticola.*

The program used to process the data and obtain the statistic calculations was the SPSS (Statistical Package for the Social Sciences) version 15.

RESULTS

The age of the patients analyzed varied between 30 and 59 years, with an mean age of 41.83 years. The two highest percentages corresponded to patients between 30 and 40 years old (44.8%) and 41 and 50 years old (44.8%), and more than half were females (58.6%).

Out of the 29 patients that took part in the research, 19 (65.5%) presented the genotype A/A, and 10 (34.5%) presented the genotype A/O, none of the patients presented the genotype O/O. It was possible to verify that the allele A prevailed (82.8%), when compared to the allele O (17.2%), as shown in Table 2.

Table 2:

Allelic frequency.

ALLELES	n	%
A	48	82,8
O	10	17,2
TOTAL	**58**	**100,0**

In table 3 are presented the results of the study of association between bacteria (Pg, Td, Tf and Aa) and presence of polymorphism of the MBL2 gene. The great majority of patients with the A/O genotype presented high percentage of Pg, Td

and Tf bacteria, with exception of Aa. The biggest difference in percentage between patients who had or didn't have polymorphism in relation to the bacteria occurred on the bacteria Aa, which was 34.2% higher in the absence of polymorphism (84.2% x 50.0%); however, when considered the margin of error used (5.0%), a significant association between the presence of polymorphism and any of the bacteria could not be proved (p > 0.05).

The percentage of patients who presented polymorphism in the MBL2 gene and presented the three bacteria (Pg, Tf and Td) was higher than the ones who didn't have polymorphism (60.0% x 52.6%); however, a significant association between the condition of polymorphism and the positivity for the three bacteria mentioned was not proved (p > 0.05).

DISCUSSION

It is well established that mutations on the exon 1 and in the promoter region of the MBL2 gene cause a variation on the protein's serum concentration; jeopardizing its immune functions against infectious agents[16]. Therefore, understanding the molecular bases for the different responses to each disease can improve our understanding about the pathogenesis of the infections and help on the treatment and control of those infections[17].

Table 3:

Association of the occurrence of bacteria in the diseased sites with the polymorphism of the MBL2 gene.

MBL2							
BACTERIA	**GENOTYPE A/A**		**GENOTYPE A/O**		**TOTAL GROUP**		**P VALUE**
	n	%	n	%	n	%	
Aa							
Positive	16	84.2	5	50.0	21	72.4	p(1) = 0.083
Negative	3	15.8	5	50.0	8	27.6	
Pg							
Positive	13	68.4	8	80.0	21	72.4	p(1) = 0.675
Negative	6	31.6	2	20.0	8	27.6	
Tf							
Positive	13	68.4	9	90.0	22	75.9	p(1) = 0.367
Negative	6	31.6	1	10.0	7	24.1	
Td							
Positive	16	84.2	8	80.0	24	82.8	p(1) = 1.000
Negative	3	15.8	2	20.0	5	17.2	
Pg + Tf + Td							
Yes	10	52.6	6	60.0	16	55.2	p(1) = 1.000
No	9	47.4	4	40.0	13	44.8	
TOTAL	**19**	**100.0**	**10**	**100.0**	**29**	**100.0**	

(1) Fisher's exact test.

Homozygous subjects for the wild type (A/A) represent most of the population; followed by heterozygous (A/O) and, finally, the genotype O/O, which covers a small part of the population[18,19]. In this study, the genotype A/A was also prevailing, followed by the genotype A/O.

In the current literature, few papers have analyzed the correlation between polymorphism of the MBL2 gene and periodontitis. Louroupoulou et al[20] didn't find an association between the polymorphism of the MBL2 gene and the susceptibility to periodontitis in the studied population.

Maffei et al[6] also tried to analyze the plasmatic levels of MBL2 in relation to the periodontitis and showed that the levels of this protein were not high on patients who had periodontal disease and that the deficiency of MBL2 is not related to the severity and susceptibility to periodontitis.

In a study with a diabetic Brazilian population, Araújo et al[2] verified that less than half of the patients with periodontitis presented the genotype A/A and most had the genotype A/O; not having a significant statistic difference between patients with periodontitis and the control group when it comes to genotipic frequency of the MBL2. Similar results were observed in this study and also found in other studies[18,19] where the wild genotype (A/A) prevailed in patients with chronic periodontitis.

Contrary to the current study, Tsutsumi et al[21] verified that the frequency of the A/O genotype was little higher on patients with chronic periodontitis.

The periodontal disease, being one of the most common multibacterial pathologies, can be a useful model for studying the interaction between microbes and genetic[17]. Nibali et al[7] studied the presence of bacteria in periodontal pockets associated with the polymorphism of genes that code receptors and mediators related to inflammation, and revealed that these polymorphisms are associated with a higher chance of detecting Aa, Pg and Tf in patients with aggressive periodontitis.

When relating to the allele frequency, it was observed in this study that the wild A allele prevailed, this data being in accordance with the findings of Araújo et al[2] and Tsutsumi et al,[17] who also verified a higher percentage of the wild allele's presence.

In general, one third of the white population is considered MBL deficient; however, the threshold of this deficiency is still subject of debate[6]. Furthermore, the affinity of the binding between MBL and carbohydrates in the microorganisms' surface is variable, which can be classified as high, moderate, light or absent — these variations occur either among different groups of microorganisms, or even within the same species[1].

More studies to relate genetic polymorphism and the developing of periodontal disease are necessary, for in the periodontitis, besides the infection by

periodontal pathogens, the host and environmental factors have an important role in the susceptibility and severity of the disease[6]. Besides, it is important to emphasize that the miscegenation of the Brazilian population favors the genetic heterogeneity, which complicates the genetic mapping; as the ethnic origin of the Brazilian population, especially in the northeast region, which is mostly composed by Africans, followed by Caucasians and, least by native Americans; which characterizes a great mixture of genotypes[22].

CONCLUSION

This study revealed that there was no significant association between the found genotypes and the presence the periodontopathogens Aa, Pg, Tf and Td in patients who have chronic periodontitis from the Brazil's northeast population. Before what was described here, new studies can be developed from this methodology, however, with a better availability of time and resources to increase the number of the sample and also evaluate other genes related to immunity and to chronic periodontitis. Thereby, it is expected that scientific breakthrough may lead to genetic markers that helps professionals understand, not only known factors, but the different patterns presented by periodontal disease; including different responses for treatments already established, and to plan in a more indicated way a better conduct for each patient.

ACKNOWLEDGMENTS

To the financial support given by CNPq (*Conselho nacional de desenvolvimento científico e tecnológico*) and FACEPE (*Fundação de amparo à ciência e tecnologia de Pernambuco*), through the undergraduate research scholarship.

REFERENCES:

1. Furtado VC. Perfil do polimorfismo gênico da b-defensina-1 e da MBL2 em pacientes com diagnóstico de onicomicose causadas por Candida spp [tese]. Recife (PE): Universidade Federal de Pernambuco; 2008.
2. Araújo NC, Bello DMA, Souza PR, Cimões R. Association of polymorphism of the mbl2 gene in type 2 diabetic patients with periodontitis: a preliminary study. Acta Stomatol Croat. 2009;43(4):290-300.
3. Bravo F. Associação entre a periodontite e o polimorfismo genético da lectina de ligação da manose (MBL) em uma população brasileira [tese]. Recife (PE): Universidade Federal de Pernambuco; 2010.
4. Arraes LC, Souza PR, Bruneska D, Castelo Filho A, Cavada BS, Lima Filho JL, et al. A cost-effective melting temperature assay for the detection of single nucleotide polymorphism in the MBL2 gene of HIV-1-infected children. Braz J Med Biol Res. 2006;39(6):719-23.
5. Turner MW. The role of mannose-binding lectin in health and disease. Mol Immunol. 2003 Nov;40(7):423-9.
6. Maffei G, Brouwer N, Dolman KM, Van Der Velden U, Roos D, Loos BG. Plasma levels of mannan-binding lectin in relation to periodontitis and smoking. J Periodontol. 2005 Nov;76(11):1881-9.
7. Carvalho C, Cabral CT. Papel da Porphyromonas gingivalis na doença periodontal. Rev Port Estomatol Cir Maxilofac. 2007;48(3):167-71.
8. Querido SMR, Dotto PP, Aquino DR, Cortelli JR. Aspectos clínicos, radiográficos e microbianos de uma família com expressiva prevalência de doença periodontal. Rev Odonto Ciência. 2006;21(52):163-71.
9. Nuto SAS, Nations MK, Costa ICC. Aspectos culturais na compreensão da periodontite crônica: um estudo qualitativo. Cad Saúde Pública. 2007;23(3):681-90.
10. Americam Academy of Periodontology. Periodontitis. Ann Periodontol. 1999;4:32-7.
11. American Academy of Periodontology. Parameter on chronic periodontitis with slight to moderate loss of periodontal support. J Periodontol. 2000;71(5 Suppl):853-5.
12. Ávila-Campos MJ, Velásquez-Meléndez G. Prevalence of putative periodontopathogens from periodontal pacients and healthy subjects in São Paulo, SP, Brazil. Rev Inst Med Trop Sao Paulo. 2002; 44: 1-5.
13. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol. 1996 Aug;11(4):266-73.
14. Minchinton RM, Dean MM, Clark TR, Heatley S, Mullighan CG. Analysis of the relationship between mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. Scand J Immunol. 2002 Dec;56(6):630-41.
15. Summerfield JA, Ryder S, Sumiya M, Thursz M, Gorchein A, Monteil MA, et al. Mannose-binding protein gene mutations associated with unusual and severe infections in adults. Lancet. 1995 Apr 8;345(8954):886-9.
16. Araújo MSM. Investigação do polimorfismo do éxon-1 do gene MBL (Manose-Binding Lectin) em pacientes portadores de tuberculose [tese]. Belém (PA): Universidade Federal do Pará; 2009.
17. Nibali L, Ready DR, Parkar M, Brett PM, Wilson M, Tonetti MS, et al. Gene Polymorphisms and the Prevalence of Key Periodontal Pathogens. J Dent Res. 2007 May;86(5):416-20.
18. Kalil AC. Can Serum Mannose-Binding Lectin Levels Aid with the Diagnosis of Invasive Aspergillosis? Clin Infect Dis. 2009 Nov 15;49(10):1492-5.
19. Mead R, Jack D, Pembrey M, Tyfield L, Turner M. Mannose-binding lectin alleles in a prospectively recruited UK population. Lancet. 1997 June 7;349(9066):1669-70.
20. Louropoulou A, Van der Velden U, Schoenmaker T, Catsburg A, Savelkoul PHM, Loss BG. Mannose-binding lectin gene polymorphisms in relation to periodontitis. J Clin Periodontol. 2008 Nov;35(11):923-30.
21. Tsutsumi A, Kobayashi T, Ito S, Goto D, Matsumoto I, Yoshie H, et al. Manose binding lectin gene polymorphism and th severity of chronic periodontitis. Nihon Rinsho Meneki Gakkai Kaishi. 2009 Feb;32(1):48-52.
22. Alves-Silva J, Santos MS, Guimarães PEM, Ferreira ACS, Bandelt H, Pena SDJ, et al. The ancestry of Brazilian mtDNA lineages. Am J Hum Genet. 2000 Aug;67(2):444-61.

How to cite: Cimões R, Souza PRE, Vajgel BCF, Dias RSAM, Ferreira B. The presence of periodontal pathogens and their relationship with the MBL2 gene in patients with chronic periodontitis. J Clin Dent Res. 2018 May-Aug;15(2):170-9.

Submitted: May 25, 2016 - Revised and accepted: May 15, 2018.

Contact address: Renata Cimões Jovino Silveira
Pós-Graduação em Odontologia - Avenida Prof. Moraes Rego 1235 - Cidade Universitária, CEP: 50.670-901. Recife/PE, Brazil
E-mail: renata.cimoes@globo.com

» The authors report no commercial, proprietary or financial interest in the products or companies described in this article.

» Patients displayed in this article previously approved the use of their facial and intraoral photographs.