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Original article

Association of systemic beta-defensin-1 and -20G/A DEFB1 gene polymorphism with Behçet's disease

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ABSTRACT

Background: Behçet's disease (BD) is a multisystem inflammatory disease of unknown etiology. Beta-defensins are antimicrobial peptides involved in epithelial host defense. To explore whether beta-defensins might be involved in BD pathogenesis, we examined plasma human beta-defensin-1 (hBD-1) and DEFB1 -20G/A polymorphism in BD patients.

Methods: This case-control study included 106 BD patients fulfilling the criteria of the International Study Group for BD and 156 controls. The -20G/A genotypes were determined by PCR-RFLP analysis in all participants, and plasma hBD-1 was assessed by ELISA in 77 BD patients and 44 controls, only. Stepwise multiple regression models were applied to determine independent predictors for plasma hBD-1 in BD patients.

Results: Distribution of -20G/A genotypes was different between BD patients and controls. Compared to GG genotype, "GA" genotype [OR (95% CI), 3.12 (1.56–6.16); p = .001] and "AA" genotype [2.57 (1.10–5.96); p = .027)] were associated with increased risk for BD. Plasma hBD-1 concentrations were significantly higher in BD patients than controls (9.81 ± 3.52 ng/mL *vs.* 5.30 ± 3.02 ng/mL; p < .001), and in BD patients with neurological involvement than those without (11.1 ± 4.12 ng/mL *vs.* 9.19 ± 3.10 ng/mL; p = .040). No variation was noted according to other clinical features, treatment received or -20G/A genotypes. In multivariate analysis, neurological involvement was the only predictor for plasma hBD-1 (β, 0.274; p = .029).

Conclusions: Findings suggest that hBD-1 and its encoding gene DEFB1 could modulate the risk for BD, especially for BD neurological involvement. Further work is needed for a better understanding of role of hBD-1 and its genetic variants in the pathogenesis of BD.

1. Introduction

Behçet's disease (BD) is a multisystem inflammatory disorder of unclear etiology, characterized by unpredictable periods of recurrences and remissions, although the frequency and severity tend to abate with time. Skin-mucosa lesions especially oral ulcers are the most common manifestations and are usually the presenting symptoms, whereas ocular, vascular and neurological involvements are less common but more serious [1]. Other manifestations include arthritis, a positive pathergy test, thrombophlebitis, and gastrointestinal ulcerations. No pathognomonic laboratory findings exist, and then diagnosis of BD is based on clinical criteria though important limitations have been noted [2,3]. BD has a worldwide distribution; however, it is most prevalent along the ancient Silk Road linking Rome with China. The prevalence is the highest in Turkey, followed by the countries bordering the Mediterranean Sea and the Middle and Far East [4]. The predominant histopathological feature of BD is vasculitis with singular properties among other systemic vasculitis manifestations, as it can involve all veins, arteries and vessels of all sizes. Although Behçet syndrome is increasingly being recognized, investigated and effectively managed, its etiology and pathogenesis remains unclear. It's thought to be due to aberrant immune activity triggered by exposure to infectious or environmental agents in genetically predisposed individuals [5,6]. Ethnic and regional differences in BD presentation are well known. Not only

Abbreviations: BD, Behcet's disease; hBD-1, human beta-defensin-1

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the frequency but also organ lesions seem to differ between regions. These differences could be related to genetic and environmental influences. This is why some prefer to see in BD a syndrome rather than a disease [7].

Attention has been focused on endogenous antimicrobial peptides called β-defensins as a component of the innate immune system against microorganisms. These peptides, mainly produced by epithelial cells, exert multiple functions including antimicrobial and immunomodulatory activities [8]. One member of this family, human β-defensin-1 (hBD-1), which is constitutively expressed, has the ability to kill or inactivate a wide spectrum of bacteria and fungi by altering their membranes stability [9,10]. The DEFB1 gene that encodes the hBD-1 is located in region p23 of chromosome 8 [11]. It is expressed in epithelial cells in several tissues and in different immune cells [12,13]. Single-nucleotide polymorphisms (SNPs) in DEFB1 gene have been associated with human diseases affecting mucous membranes, such as periodontitis [14], allergic rhinitis [13] and bronchial asthma [15]. This suggests that hBD-1 plays a key role as a host defense at mucosal surfaces. Since BD is mainly expressed by mucosal lesions with oral aphthosis as a constant symptom and since it is produced by oral mucosa and salivary glands [16], altered production or expression of this antimicrobial peptide might be involved in the pathophysiology of BD. However, only few studies have evaluated β-defensins in BD. In this study, we investigated the possible association between -20G/A DEFB1 gene polymorphism and systemic hBD-1 with BD in Tunisian population.

2. Material and methods

2.1. Subjects

Patients diagnosed with BD and regularly followed and treated in the department of internal medicine at Rabta Hospital (Tunis, Tunisia) were invited to participate in the study. Diagnosis of BD in all patients was based on the criteria of the International Study Group for Behçet's Disease [2]. Controls were employees (doctors, nurses, workers and trainees) of Rabta hospital and their relatives. They were selected by matching to patients by gender, five-year age class and neighborhood income. Criteria of eligibility for both patients and controls were to be free of past or current inflammatory, autoimmune or systemic disease (except BD for patients), malignancy, renal or liver function impairment and current pregnancy. After applying of these criteria, 106 patients with BD and 156 controls were retained for analysis. The flow diagram of study participants is shown in Fig. 1. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The protocol was approved by the Ethics Committee of Rabta hospital and all subjects gave written informed consent.

2.2. DNA analysis

Genomic DNA was extracted from EDTA anticoagulated whole blood using conventional salting-out procedure [17]. Genotyping of -20G/A DEFB1 polymorphism (rs11362) was carried out by PCR-RFLP method as previously described [18]. The sequences of primers were 5′-GACCAGGTTGTGCAATCCACCAG-3′ (forward primer) and 5′-GCAG AAGGTAGGAAGTTCTCATGGCG-3′ (reverse primer). PCR reactions were performed on a total volume of 50 µL using 400 ng of genomic DNA, 25 pmol of each primer, 200 µM dNTPs and 1.5 U Taq polymerase (Promega, Madison, WI) in PCR buffer with 2.5 mM MgCl2. The PCR program was a denaturating step at 95 °C for 10 min followed by 34 cycles of 95 °C for 15 s, annealing at 65 °C for 60 s and elongation at 72 °C for 60 s with a final elongation step at 72 °C for 2 min. PCR products were digested overnight at 37 °C with 10 U of *Dde*I restriction enzyme. This enzyme can reveal the presence of an A or G nucleotide at the -20 position. Electrophoresis analysis of digested PCR products was performed in 3% agarose gel stained with ethidium bromide to show patterns for the three genotypes: To ensure that the genotyping was adequate quality, all gels were reread blindly by 2 persons without any change, and 20% of the analyses were repeated randomly.

2.3. Measurement of plasma human β-defensin-1 concentration

Fasting venous blood samples were drawn on EDTA-containing tubes. After centrifugation at 2000 ×*g*, plasma was stored at −70 °C until analysis (within 6 months). Plasma hBD-1 concentrations were measured in duplicate on a micro plate reader (BioTek, Winooski, VT) using commercially ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For financial constraints, plasma hBD-1 was measured in 77 BD patients and 44 controls, only. There were no differences in clinical characteristics and treatment received between BD patients having or not data for plasma hBD-1.

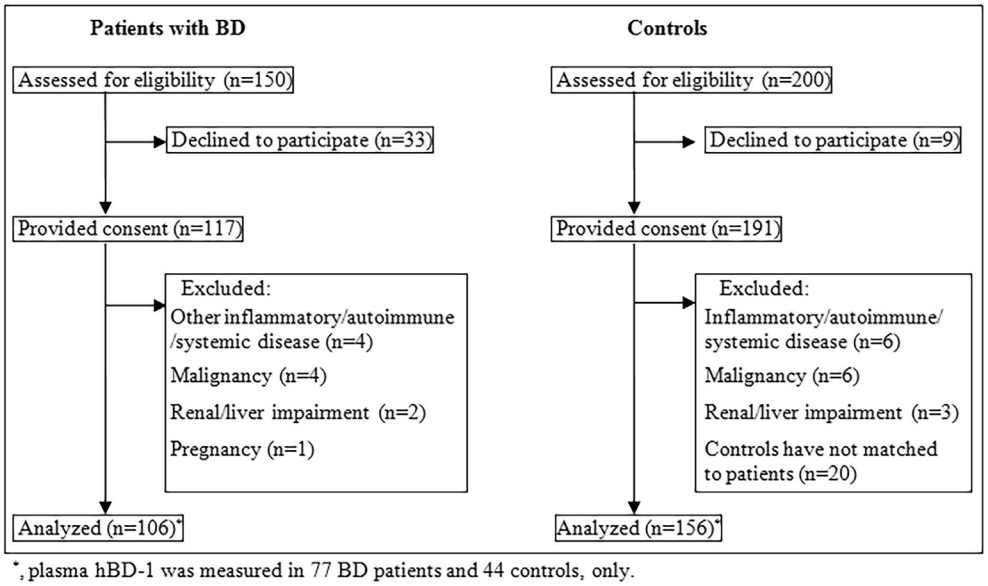

Fig. 1. Flow diagram of study participants.

Table 1
Main clinical features and treatment received in Behçet's disease patients.

Variable	Frequency
Clinical features	
Oral aphthosis	100%
Genital aphthosis	78%
Pseudofolliculitis	73%
Positive pathergy test	65%
Erythema nodosum	19%
Arthritis	46%
Vascular involvement	37%
Ocular involvement	31%
Neurological involvement	27%
Treatment received	
Colchicines	100%
Corticosteroids	75%
Azathioprine	45%
Cyclophosphamide	37%
Vitamin K antagonists	25%

2.4. Statistical analysis

Analysis of data was performed using SPSS 18.0 for Windows software (SPSS Inc., Chicago, IL). Fig. 1 was plotted using Prism software version 6.01 (GraphPad software, La Jolla, CA). Normality of distribution of hBD-1 values was confirmed by Kolmogorov-Smirnov test. Continuous variables were expressed as mean ± SD and compared with unpaired Student's *t*-tests or ANOVA. Genotypes and allelic frequencies were estimated by gene counting and were compared using chi squared tests. Unadjusted and age- and gender-adjusted odd-ratios and 95% confidence intervals were calculated using unconditional logistic regression to test association of genotypes and alleles with BD. Hardy-Weinberg equilibrium was tested using SPSS and was recalculated using Arlequin software [19]. Stepwise multiple regression models were performed to establish independent predictors for plasma hBD-1. Covariates were disease duration, arthritis, vascular, ocular and neurological involvement and treatment with corticosteroids, azathioprine, cyclosphosphamide and vitamin K antagonists, and -20G/A polymorphism. A p-value < .05 was considered statistically significant.

3. Results

There were no differences between patients and controls for age (37.8 ± 10.5 *vs.* 36.8 ± 6.94 years; p = .427) and sex-ratio (1.94 *vs.* 1.89; p = .913). Age of onset BD varied from 8 to 51 years with a median of 30 years, and disease duration ranged between one and 29 years with a median of 7 years. Main characteristics of BD patients are shown in Table 1.

Genotype and allele frequencies of DFEB1 -20G/A polymorphism in BD patients and controls are shown in Table 2. No significant deviation from the Hardy-Weinberg equilibrium was detected in either BD patients (χ^2 = 4.91; p = .086) or controls (χ^2 = 3.34; p = .188). The distribution of DFEB1 -20G/A genotypes significantly differed between BD patients and controls. The “AA” genotype and “A” allele were more

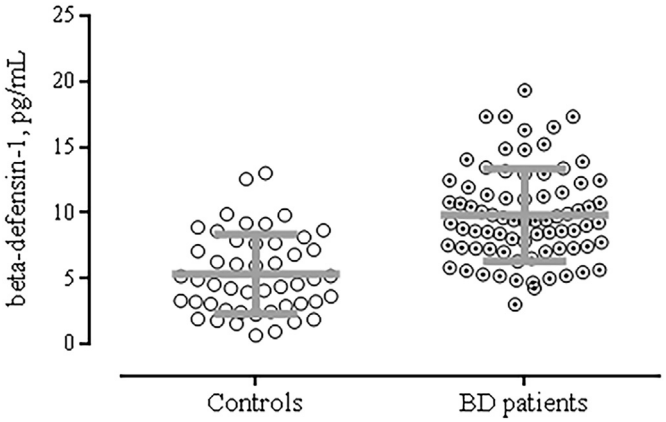

Fig. 2. Comparative distribution of plasma β-defensin-1 (hBD-1) concentrations in Behçet's disease (BD) patients and controls.
Drawbars represent the mean ± 1 standard deviation.

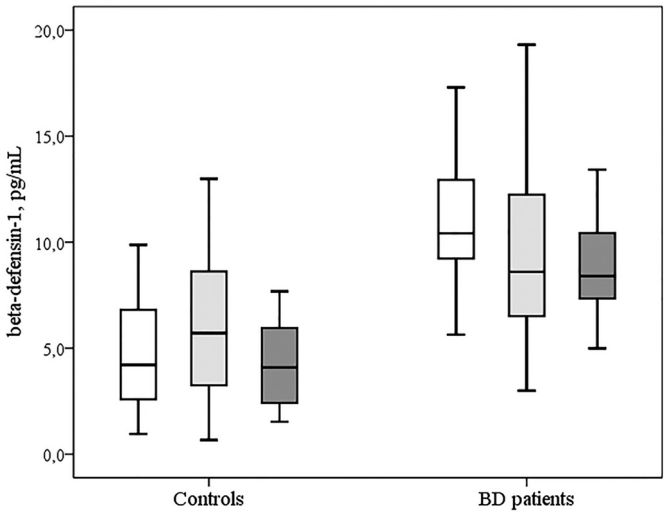

Fig. 3. Plasma β-defensin-1 (hBD-1) concentrations according to -20G/A DEFB1 genotypes in Behçet's disease (BD) patients and controls (white boxes for AA genotype; light gray boxes for AG genotype and dark gray boxes for GG genotype).
The bottom and top of the box are the first and third quartiles (inter quartile range, IQR) and the band inside the box is the median. The ends of the whiskers are the lowest and the highest values still within 1.5 IQR.

Table 2
Comparative genotypes distribution and allele frequency of BEFB1 -20G/A polymorphism in Behçet's disease (BD) patients and controls.

	Controls (n = 156)	BD patients (n = 106)	Unadjusted OR (95% CI)	Age- and sex-adjusted OR (95% CI)*
Genotype, n (%)				
GG[a]	77 (49.4)	34 (32.1)	–	–
GA	58 (37.2)	49 (46.2)	1.91 (1.09–3.33)*	3.15 (1.60–6.23)**
AA	21 (13.5)	23 (21.7)	2.48 (1.21–5.07)*	2.58 (1.11–6.00)*
Allele (%)				
G[a]	67.8	55.2	–	
A	32.2	44.8	1.72 (1.18–2.51)**	

OR, odd-ratio; CI, confidence interval.
[a] Reference category.
* p < .05.
** p < .01.

frequent in BD patients than controls (Table 1). Plasma hBD-1 concentrations were significantly higher in BD patients than controls (9.81 ± 3.52 ng/mL *vs.* 5.30 ± 3.02 ng/mL; p < .001) (Fig. 2). However, no significant difference of plasma hBD-1 was found according to the -20G/A genotypes in both patients and controls groups (Fig. 3). Plasma hBD-1 concentrations were significantly higher in patients with than those without neurological involvement (11.1 ± 4.12 ng/mL *vs.* 9.19 ± 3.10 ng/mL; p = .04). Patients who received corticosteroids during the disease course showed a trend to significant increase in plasma hBD-1 (10.1 ± 3.41 ng/mL *vs.* 8.47 ± 3.53 ng/mL; p = .09). However, no differences were found according to arthritis, vascular or ocular involvement, and treatment with azathioprine, cyslosphosphamide or vitamin K antagonists. In multivariate analysis, neurological involvement was the only independent predictor for plasma hBD-1 (β, 0.27; p = .029).

4. Discussion

This study showed higher prevalence of “A” allele of DEFB1 -20G/A polymorphism and increased plasma hBD-1 in BD patients compared to controls. Moreover, plasma hBD-1 was higher in patients with neurological involvement. These data suggest that DEFB-1 could be involved in genetic susceptibility for BD, and that systemic hBD-1 could be a marker for neurological involvement in BD.

Different SNPs in DEFB1 gene has been associated with risk for inflammatory or immune diseases such as periodontisis [14], allergic rhinitis [13], asthma [15], atopic dermatitis [20], systemic lupus erythematosus [21] and Crohn's disease [22]. Only few studies have investigated variants of β-defensins genes in BD. DEFB1 haplotypes were associated with the disease in Turkish population [23]. Copy numbers polymorphism in DEFB4 gene was associated with risk for BD in Iraqi population [24] but not in Korean population [25].

The present study showed increased systemic hBD-1 concentrations in BD patients. Mechanisms of the rise and its cellular sources are unidentified and whether it results from or contributes to the disease is unclear. Previous reports described increased circulating hBD-1 in cirrhosis [26] and over expression of the peptide in brain in Alzheimer's disease [27] and in inflamed salivary gland [28]. Thus, even being constitutively expressed, hBD-1 seems capable of up regulation upon inflammatory stimuli. The rise in circulating hBD-1 may have resulted from hyper production by epithelial or immune cells due to proinflammatory environment associated with BD. Indeed, the disorder is characterized by infiltration of neutrophil and mononuclear cells and secretion of proinflammatory cytokines [6], which are potential sources and stimuli of β-defensins production [29]. Raised hBD-1 in patients with neurological involvement, a condition more prone to inflammation, supports this option. In this line, hBD-1 levels were higher in patients taking corticosteroids or immunosuppressive treatment. But, the difference was borderline for corticosteroids only, and no longer significant in multivariate analysis. This however does not exclude that defects in antimicrobial capacity of β-defensins take place in DB.

The study showed no association of plasma hBD-1 with -20A/G polymorphism. This however doesn't exclude that the polymorphism affects gene expression. Indeed, genetic variants of DEFB1 were shown to influence peptide expression [15,30]. Also, systemic level does not always reflect efficiently tissue expression, especially for a peptide as hBD-1 that acts locally at epithelial level [26]. In this study, the rise in systemic hBD-1 in BD patients could be due to other functional polymorphisms of the gene.

This was a mono centric study and the sample size was small, particularly for the analysis of the gene polymorphism. Limited number of participants in each genotype group could have weakened the study power to detect significant association with plasma hBD-1. We focused on a single polymorphism in DEFB1 gene. Though, investigating multiple SNPs with examining combined haplotypes could bring more comprehensive information on role of DEFB genes in modulating the risk for BD. Transversal design of the study prevents establishing causal relationship between circulating hBD-1 and BD. The study did not investigate other inflammatory markers or tissue peptide/mRNA expression. Whether BD was active or quiescent at the moment of blood draw was not specified. If available, these data would have allowed a better understanding of the mechanisms of plasma hBD-1 rise in BD patients. Future work must go beyond all these limitations. Since BD is mainly expressed by oral lesions, it would be useful to explore β-defensins in saliva or mRNA expression in oral mucosa.

In conclusion, the study findings suggest that hBD-1 and DEFB1 -20G/A polymorphism are involved in BD pathophysiology. The genetic variant may be a marker for susceptibility to BD and systemic hBD-1 may be a marker for neurological involvement in BD. Replication of our findings in other populations and ethnicities will be necessary for corroborating the association and ensuring generalisability of the data. Additional work is needed to better clarify the role of β-defensins and their genetic variants in BD pathophysiology.

Conflict of interest

All authors declare no conflict of interests.

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References

[1] Yazici H, Seyahi E, Hatemi G, Yazici Y. Behçet syndrome: a contemporary view. Nat Rev Rheumatol 2018;14:107–19.
[2] International Study Group for Behçet's disease. Criteria for diagnosis of Behçet's disease. Lancet 1990;335:1078–80.
[3] Davatchi F, Assaad-Khalil S, Calamia KT, Crook JE, Sadeghi-Abdollahi B, Schirmer M, et al. International team for the revision of the international criteria for Behçet's disease (ITR-ICBD). The international criteria for Behçet's disease (ICBD): a collaborative study of 27 countries on the sensitivity and specificity of the new criteria. J Eur Acad Dermatol Venereol 2013;28:338–47.
[4] Mahr A, Maldini C. Epidemiology of Behçet's disease. Rev Med Interne (Paris) 2014;35:81–9.
[5] Houman MH, Bel Feki N. Pathophysiology of Behçet's disease. Rev Med Interne (Paris) 2014;35:90–6. https://doi.org/10.1016/j.revmed.2013.10.012.
[6] Greco A, De Virgilio A, Ralli M, Ciofalo A, Mancini P, Attanasio G, et al. Behçet's disease: new insights into pathophysiology, clinical features and treatment options. Autoimmun Rev 2018;17:567–75. https://doi.org/10.1016/j.autrev.2017.12.006.
[7] Yurdakul S, Hamuryudan V, Yazici H. Behcet syndrome. Curr Opin Rheumatol 2004;16:38–42.
[8] Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. Cell Mol Life Sci 2006;63:1294–313.
[9] Huang YP, Wang TY, Wang W, Sun HZ. Association between genetic polymorphisms in DEFB1 and susceptibility to digestive diseases. Med Sci Monit 2015;21:2240–50.
[10] Silva O, Porto W, Ribeiro S, Batista I, Franco O. Host-Defense peptides and their potential use as biomarkers in human diseases. Drug Discov Today 2018;23:1666–71. https://doi.org/10.1016/j.drudis.2018.05.024.
[11] Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human β-defensin-1 is a salt-sensitive antibiotic in the lung that is inactivated in cystic fibrosis. Cell 1997;88:553–60.
[12] Kallsen K, Andersen E, Heine H. Histone deacetylase (HDAC)1 controls the expression of beta defensin 1 in human lung epithelial cells. PloS One 2012;7:e50000. https://doi.org/10.1371/journal.pone.0050000.
[13] Bogefors J, Kvarnhammar AM, Höckerfelt U, Cardell LO. Reduced tonsillar expression of human β-defensin 1, 2 and 3 in allergic rhinitis. FEMS Immunol Med Microbiol 2012;65:431–8. https://doi.org/10.1111/j.1574-695X.2012.00959.x.
[14] Schaefer AS, Richter GM, Nothnagel M, Laine ML, Ruhling A, Schafer C, et al. A 3’ UTR transition within DEFB1 is associated with chronic and aggressive periodontitis. Genes Immun 2010;11:45–54. https://doi.org/10.1038/gene.2009.75.
[15] Zaitseva MA, Bragvadze BG, Svitich OA, Namazova-Baranova LS, Gankovskaya LV. Analysis of TLRs genes expression and DEFB1 polymorphisms association in children with bronchial asthma. Bull RSMU 2016(3):39–43.
[16] Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Microsc 2003;36:247–52.
[17] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;11(16):1215.
[18] Prado-Montes de Oca E, Rangel-Villalobos H, Gallegos-Arreola MP, Sandoval L, Figuera LE. SNPs in human beta-defensin 1 gene (DEFB1): frequencies in a Mexican population and new PCR-RFLPs assays. Int J Immunogenet 2006;33:339–42.
[19] Schneider S, Roessli D, Excoffier L. Arlequin Version 2.000: Software for Population

Genetics Data Analysis. Switzerland: Genetics and Biometry Laboratory, University of Geneva; 1995-2000.
[20] Prado-Montes de Oca E, Garcia-Vargas A, Lozano-Inocencio R, Gallegos-Arreola MP, Sandoval-Ramírez L, Dávalos-Rodríguez NO, et al. Association of β-defensin 1 single nucleotide polymorphisms with atopic dermatitis. Int Arch Allergy Immunol 2007;142:211–8.
[21] Sandrin-Garcia P, Brandão LA, Guimarães RL, Pancoto JA, Donadi EA, Lima-Filho JL, et al. Functional single-nucleotide polymorphisms in the DEFB1 gene are associated with systemic lupus erythematosus in southern Brazilians. Lupus 2012;21:625–31.
[22] Kocsis AK, Lakatos PL, Somogyvári F, Fuszek P, Papp J, Fischer S, et al. Association of beta-defensin 1 single nucleotide polymorphisms with Crohn's disease. Scand J Gastroenterol 2008;43:299–307.
[23] Ceren Karahan Z, Biriken D, Boyvat A, Dolapei I, Asku E, Ozenci H. Functional polymorphisms and haplotypes of human Beta Defensin 1 (DEFB1) gene in Behcet's disease patients. Clin Exp Rheumatol 2010;28:S164.
[24] Hameed AF, Jaradat S, Al-Musawi BM, Sharquie K, Ibrahim MJ, Hayani RK, et al. Association of higher defensin beta-4 genomic copy numbers with Behçet's disease in Iraqui patients. Sultan Qaboos Univ Med J 2015;15:491–5.
[25] Park JJ, Oh BR, Kim JY, Park JA, Kim C, Lee YJ, et al. Copy number variation of β-defensin genes in Behçet's disease. Clin Exp Rheumatol 2011;29(4):S20–3. Suppl 67.
[26] Kaltsa G, Bamias G, Siakavellas SI, Goukos D, Karagiannakis D, Zampeli E, et al. Systemic levels of human β-defensin 1 are elevated in patients with cirrhosis. Ann Gastroenterol 2016;29:63–70.
[27] Williams WM, Torres S, Siedlak SL, Castellani RJ, Perry G, Smith MA, et al. Antimicrobial peptide β-defensin-1 expression is upregulated in Alzheimer's brain. J Neuroinflammation 2013;10:127https://doi.org/10.1186/1742-2094-10-127.
[28] Sahasrabudhe KS, Kimball JR, Morton TH, Weinberg A, Dale BA. Expression of the antimicrobial peptide, human beta-defensin 1, in duct cells of minor salivary glands and detection in saliva. J Dent Res 2000;79:1669–74.
[29] Prado-Montes de Oca E. Human beta-defensin 1: a restless warrior against allergies, infections and cancer. Int J Biochem Cell Biol 2010;42:800–4. https://doi.org/10.1016/j.biocel.2010.01.021.
[30] Polesello V, Zupin L, Di Lenarda R, Biasotto M, Ottaviani G, Gobbo M, et al. Impact of DEFB1 gene regulatory polymorphisms on hBD-1 salivary concentration. Arch Oral Biol 2015;60:1054–8. https://doi.org/10.1016/j.archoralbio.2015.03.009.