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Brza metoda za genotipiziranje DEFB1 - 44C/G SNP-a kod brazilskih pacijenata s parodontitisom

A Fast Method for DEFB1-44C/G SNP Genotyping in Brazilian Patients with Periodontitis

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Sažetak
Svrha: Defensini su kationski antimikrobni peptidi koji se pojavljuju u epitelnim stanicama. Imaju antimikrobna, antifungalna i antivirusna svojstva te su prirođena komponenta imunosnog odgovora. Jedna od hipoteza jest da peptidi štite usnu šupljinu. U ovom istraživanju procijenili smo polimorfizam gena DEFB1 kod dijabetičara s parodontitisom ili bez njega i rezultate usporedili sa zdravom kontrolnom skupinom. **Materijali i metode:** Koristili smo se testom Hairpin-Shaped Primer (HP) kako bismo istražili rasprostranjenost -44 C/G SNP-a (rsl1800972) u 119 uzoraka ljudske DNK dobivene od dijabetičara i zdravih pacijenata. **Rezultati:** Rezultati su pokazali da izmedu grupa nema razlika u rasprostranjenosti, te da je kod dijabetičara s parodontitisom češći homozigotni mutant. **Zaključak:** Potrebna su daljnja istraživanja kako bi se otkrila uloga polimorfizma DEFB1 kod dijabetičara s parodontitisom te utjecaj peptida na parodontne patogene.

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Ključne riječi
defenzini; kationski antimikrobni peptidi; DEFB1 bjelančevina, humana; epitelne stanice; dijabetes, komplikacije; parodontitis

Uvod

Ljudski beta-defensini (hBD) porodica su malih pepitda kodiranih u klasterima gena na ljudskom kromosomu 8p222-23 (Jia i suradnici, 2001.) koji pokazuju širok spektar antimikrobne aktivnosti prema gram-pozitivnim i gram- negativnim bakterijama, gljivicama i virusima. Antimikrobno djelovanje aktivira se ako se stanične membrane remete kreiranjem pora u membrani (Jurevic i suradnici, 2002.). Dio prirođenog imunosnog sustava su β-defensini, a djeluju kao prva crta obrane (Guimarães i suradnici, 2009.). Uglavnom ih proizvode epitelne stanice kože, bubrega, dušnika i bronha. Otpuštaju se nakon invazije mikroba te su locirani na mjestima na kojima je domaćin izložen okolišu, kao što su sluznica i koža (Travis i suradnici, 2001.). Unutar svih β-defensina, ljudski beta defensin 1 (hBD-1) važan je u slučaju nastanka upale (Oppenheim i suradnici, 2003.). Osim širokoga spektra antimikrobnih svojstava, postoje dokazi da hBD-1 djeluje kao kemokin na nezrele dendritičke stanice i memorijske T-stanice te tako povezuje prirođenu i stečenu imunost (Yang i suradnici, 1999.).

Introduction

human beta-defensins (hBD) are a family of small peptides, encoded by a gene cluster located on human chromosome 8p22-23 (Jia et al., 2001), exhibit a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses. The antimicrobial action is postulated to be a disruption of the microbial membrane by pore formation (Jurevic et al. 2002). β-defensins are part of the innate immune system and act as the first line of human defence (Guimarães et al., 2009) and are primarily expressed by epithelial cells of the skin, kidneys and trachea-bronquial. β-defensins are released upon microbial invasion and are located at the host environment interface, such as mucosa and skin (Travis et al., 2001). Within β-defensins the human beta defensin 1 (hBD-1) is also known to play a direct role on inflammation pathway (Oppenheim et al., 2003). In addition to their broad spectrum antimicrobial properties, there is evidence that hBD-1 act as chemokine for immature dendritic cells and memory T cells, and thus may serve as an im-

Polimorfizam jednog nukleotida (SNP) najčešći je oblik genetske varijacije između pojedinaca koje u nekim slučajevima uzrokuju bolesti (Waterfall, Cobb, 2001.) Također se rabi za identifikaciju gena uključenih u uobičajene poligenetske i multifaktorijalne bolesti (Gray i suradnici, 2000.) te služi za razumijevanje patogeneze određenih bolesti (Boniotto i suradnici, 2004.). Tri SNP-a locirana na mjestima -52G>A (rs1799946), -44C>G (rs1800972) i -20G>A (rs11362) u 5' neprevedenoj regiji (UTR) od gena DEFB1, zabilježena su kao uzročnici iste funkcionalne promjene ekspresije hBD-1 u različitim staničnim modelima (Sun i suradnici, 2006.; Milanese i suradnici, 2007.).

Transverzija C→G na mjestu -44 od 5' neprevedene regije gena DEFB1 (koji kodira ljudski β-defensin 1), povezana je u bijeloj populaciji s prisutnošću gljivice *Candida sp*. u ustima (12) te s opasnošću od infekcije virusom ljudske imunodeficijencije tipa 1 (1). Još uvijek nije pronađena poveznica za rani nastanak parodontne bolesti u dvjema različitim etničkim skupinama – bjelačkoj i crnačkoj populaciji u Americi (Boniotto i suradnici, 2004.). Tri gena 5'UTR DEFB1 SNP imaju različitu frekvenciju, što ovisi o etničkoj skupini (Kocsis i suradnici, 2008.).

Parodontitis je kronična upalna bolest koja zahvaća potporni aparat zuba (Socransky i suradnici, 1998.) i smatra se šestom komplikacijom od koje pate dijabetičari (Lim i suradnici, 2007.). Općeprihvaćena je činjenica da parodontitis uzrokuju bakterije, ali samo one nisu dovoljne za razvoj bolesti. Naime, imunosni odgovor domaćina te genetski i okolišni čimbenici određuju osjetljivost na bolest te na stupanj njezina razvoja (Kinana i suradnici, 2006.). Razvoj parodontne bolesti određen je vezom između pariopatogenog/patogenog potencijala plaka i imunosnog odgovora domaćina (Araujo i suradnici, 2009.).

Ako se uzme u obzir da je imunosni sustav važan u patogenezi parodontitisa, svrha ovog istraživanja bila je analizirati prisutnost polimorfizma gena DEFB1 na kodonu -44C/G u populaciji brazilskih pacijenata s dijabetesom tipa 2 i kroničnim parodontitisom ili bez njega, u usporedbi s parodontno i općenito zdravim pacijentima.

Materijali i metode

Uzorak istraživanja

U ovom kontroliranom istraživanju sudjelovalo je 118 ispitanika. Njih 84 bolovalo je od dijabetesa tipa 2 te su se liječili u Zavodu za endokrinologiju Sveučilišne bolnice Pernambuco. U istoj ustanovi bila su upisana i 34 zdrava ispitanika. Svi sudionici potpisali su informirani pristanak. Istraživanje je odobrilo Etičko povjerenstvo Sveučilišta Pernambuco.

Kriteriji za odabir pacijenata s dijabetesom bili su dijagnoza šećerne bolesti tipa 2 i najmanje osam vlastitih zuba. Nisu mogli sudjelovati oni koji su se liječili antibioticiportant bridge between the innate and adaptive immune systems (Yang et al., 1999).

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation between individuals; some have been observed to cause several diseases (Waterfall, Cobb 2001) and have been proven to be useful tools for identifying genes involved in common polygenic and multifactorial diseases (Gray et al., 2000). Moreover, they are useful in understanding the pathogenesis of several diseases (Boniotto et al., 2004). Three SNPs localized at positions -52G>A (rs1799946), -44C>G (rs1800972), and -20G>A (rs11362) in the 5' untranslated region (UTR) of *DEFB1* gene have been reported to promote the same functional alteration of the hBD-1 expression in different cellular models (Sun et al., 2006; Milanese et al., 2007).

The C→G transversion at position -44 of the 5' untranslated (UTR) region *DEFB1* gene (which encodes human β-defensin 1) has been associated with *Candida sp*. carriage in the mouth (12) and with the risk of human immunodeficiency virus type 1 infection in the Caucasian population (1). However, there has not been an established association for two different ethnic groups for early-onset periodontal disease, Caucasian and African-Americans (Boniotto et al., 2004). The three 5'UTR DEFB1 gene SNPs exhibit differences in frequency between ethnic groups (Kocsis et al. 2008).

Periodontitis is a chronic inflammatory disease that affects the supporting tissues of the teeth (Socransky et al., 1998) and has it been reported to be the sixth classic complication of the diabetic patient (Lim et al., 2007). It is generally accepted that the presence of bacteria is a necessary but not sufficient condition for the development of periodontitis. Host immune responses, genetic and environmental factors determine part of the susceptibility and severity of periodontitis (Kinane et al., 2006). The development of periodontal disease seems to be determined by the relationship between the periodontopathogenic potential of the plaque and the immune response of the host (Araujo et al., 2009).

Considering that the immune system plays a crucial role in the pathogenesis of periodontitis, the aim of this study was to analyze the presence of *DEFB1* gene polymorphism in codon -44 C/G in a Brazilian patient sample with type 2 diabetes mellitus with and without chronic periodontitis compared with systemically and periodontally healthy patients.

Material and Methods

Study Population

This study is a case control design with a total of 118 participants. Eighty-four cases of type 2 diabetic patients were enrolled and followed up at the Department of Endocrinology of the Federal University of Pernambuco Hospital. Thirty-four unmatched healthy control patients were also enrolled. Free and informed written consent had been obtained from all patients. The research project was approved by the Ethics in Research Committee of the Federal University of Pernambuco.

ma unatrag šest mjeseci ili su također prije šest mjeseci bili na parodontološkom tretmanu, te trudnice i dojilje. Kriteriji za uključivanje u kontrolnu skupinu bili su nepostojanje dijagnoze parodontitisa, izostanak gubitka kliničkog pričvrstka ≥ 3 mm (CAL); nije smjelo biti džepova ≥ 4 mm (PPD) te sudionik nije smio imati u medicinskoj anamnezi upisan dijabetes. Zabilježeni su bili i socijalno-ekonomski podatci o pacijentima te je obavljen stomatološki klinički pregled s pomoću milimetarske parodontološke sonde North Carolina (Trinity®, Brazil). Na svakom zubu ocijenjeno je šest mjesta za PPD-e, BOP i CAL. Parodontna bolest definirana je gubitkom pričvrstka od pet milimetara ili više, na četiri ili više mjesta, a jedno od njih trebalo je imati džep dubine četiri milimetra ili više (Becker i suradnici, 1990.).

Ekstrakcija DNK i tipizacija gena

Nakon kliničkog pregleda, prikladnim citološkim strugačem (Kolplast®, Sao Paulo, Brazil) uzet je s oralne sluznice uzorak stanica koji je nakon toga uronjen u jedan mililitar fiziološke otopine (Laboratory Tayuyna, Ltda – Nova Odessa, Sao Paulo, Brazil). Sav skupljeni materijal čuvao se na temperaturi od -20ºC za potrebe ekstrakcije DNK, koristeći se protokolom Geneclean® (GeneClean® kit, Bio 101, La Jolla, CA, SAD).

Otkrivanje polimorfizma na poziciji -44 C/G regije 5'UTR od DEFB1 provedeno je tehnikom Q-PCR i hairpin primerime (HP). Sekvencije C- i G-alela specifične za HP bile su: GGCTGGACCTCCAATGGAGCCAGCC (divlji uzorak) i CGCTGGACCTCCAATGGAGCCAGCG (mutantski uzorak). Zajednički obrnuti primer bio je CAGGATTTCAGGAACTGGGGAG, a duljina amplikona iznosila je 45-bp. Za svaki uzorak DNK rađena su dva paralelna PCR-a od 25µl – jedna C-alela specifična za HP i druga G-alela specifična za HP. Svaka reakcija sadržavala je 12,5 µl SYBR-a Green PCR Master Mix 1X (LGC, Biotechnology, Australija), 1 µM svakog primera i 10 ng predloška DNK. Sve PCR-reakcije rađene su instrumentom Rotor-Gene Q real-time PCR (Qiagen, Koeln, Njemačka), a uvjeti termalnog cirkuliranja podijeljeni su na tri stadija:

stadij 1. – 95o. C 10 min, 70o.C 30 s;
stadij 2. – 72o. C 30 s, 95o. C 20 s, 69o. C 30 s, snižavajući se prema 1o. C svaki ciklus 10 ciklusa;
stadij 3. – 72o. C 30 s, 95o. C 20 s, 60o. C 30 s tijekom 40 ciklusa.

Podatci su skupljeni tijekom zadnjeg dijela trećeg stadija kako bismo izračunali Ct svake amplifikacije krivulje. Ovu metodu opisao je 2004. godine Boniotto sa suradnicima.

Statistička analiza

Interval pouzdanosti bio je određen na 95 posto, tako da su bile prihvaćene p-vrijednosti ispod 0,05. Rezultati koji nisu dosegnuli te vrijednosti nisu se smatrali značajnima. Sve analize podataka obavljene su statističkim paketom SPSS verzije 13,0 (SPSS Inc., Chicago, IL, SAD).

Inclusion criteria for diabetic patients were: diagnosis of type 2 diabetes; having at least eight natural teeth. Exclusion criteria were: have used antibiotics for the last six months; have undergone periodontal treatment for the last six months, being pregnant or breastfeeding. Inclusion criteria for controls: no history of periodontitis, absence of any ≥ 3mm clinical attachment loss (CAL); absence of any ≥ 4mm probing pocket depth (PPD), no history of diabetes. All patients have registered socioeconomic data and underwent a clinical examination using a millimeter North Carolina-type periodontal probe (Trinity®, Brazil). Six sites per tooth were evaluated for PPD, BOP, CAL. Periodontal disease was defined as four or more sites with a loss of attachment of 5mm or more with one or more of those sites having a pocket of 4mm or more (Becker et al., 1990).

DNA extraction and Genotyping

After clinical examination, the collection of scaling cells from the oral mucosa was carried out with appropriate cytobrush-type brushes (Kolplast®, Sao Paulo, Brazil), which were subsequently stored in 1 ml of saline solution of 0.9% chloride sodium (Laboratory Tayuyna, Ltda – Nova Odessa, Sao Paulo, Brazil), the collected material was stored at -20ºC for DNA extraction and performed with Geneclean® (GeneClean® kit, Bio 101, La Jolla, CA, USA) kit following manufacturer's protocols.

The detection of polymorphism in position -44 C/G of 5'UTR region of DEFB1 was conducted by the technique Q-PCR and hairpin-shaped primer (HP). Sequences of the forward C- and G- allele specific HPs were: GGCTGGACCTCCAATGGAGCCAGC**C** (wild template) and CGCTGGACCTCCAATGGAGCCAGC**G** (mutant template), respectively. The common reverse primer was: CAGGATTTCAGGAACTGGGGAG, the length of amplicon was 45-bp. For each DNA sample two real-time PCRs of 25µl were run in parallel, one with C-allele-specific HP and the other with the G-allele-specific HP. Each reaction contained 12.5 µl SYBR Green PCR Master Mix 1X (LGC, Biotechnology, Australia), 1µM of each primer, 10ng of template DNA. All PCRs were run in a Rotor-Gene Q real-time PCR instrument (Qiagen, Cologne, Germany), thermal cycling conditions were divided in three stages as follows: stage 1 – 95o. C for 10 min, 70o.C for 30s; stage 2 – 72o.C for 30s, 95o.C for 20s, 69o.C for 30s lowering 1o.C each cycle for 10 cycles; stage 3 – 72o.C for 30s, 95o.C for 20s, 60o.C for 30s for 40 cycles. Data were collected in the last step of stage 3 in order to calculate the *Ct* of each amplification curve. This methodology was previously described (Boniotto et al., 2004).

Statistical Analysis

The confidence interval considered was 95%, so p-values less than 0.05 were accepted. The results which did not attain this value were considered to be without significance. All data analyses were performed using the statistical package SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

Rezultati

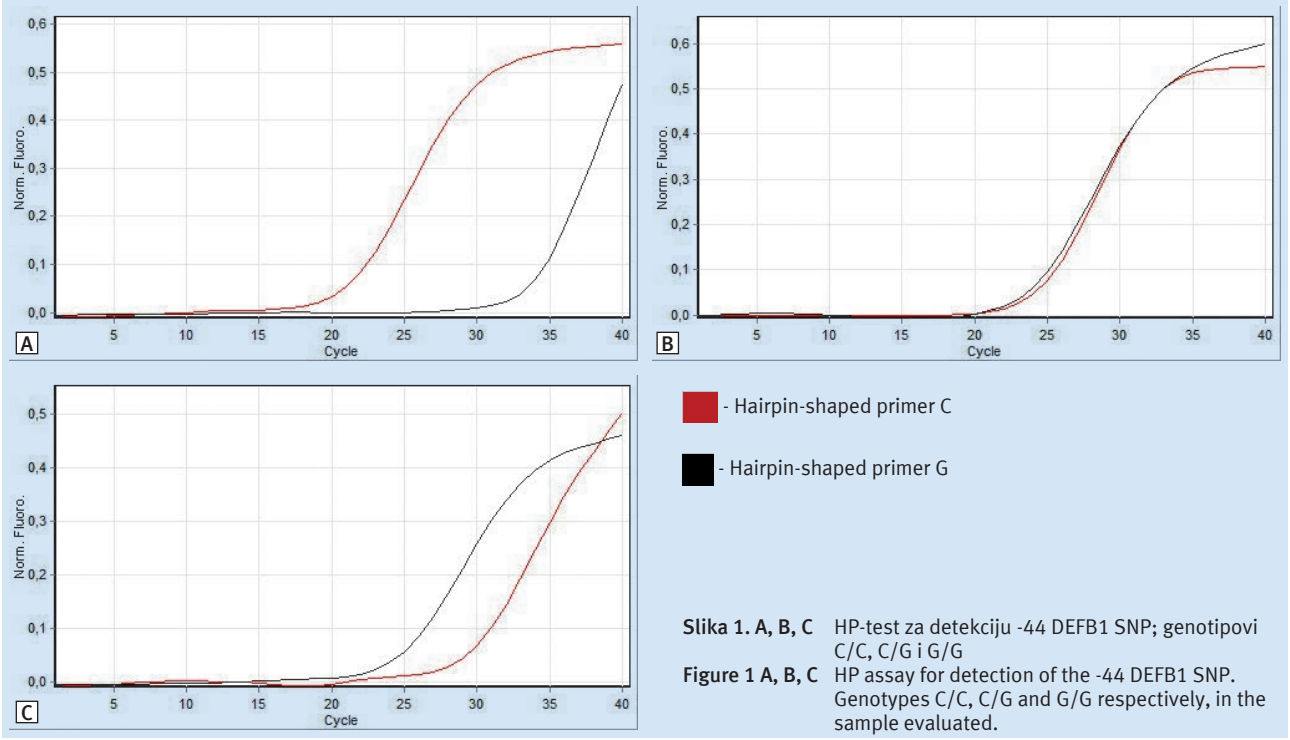
Srednja dob pacijenata iznosila je 45,5 godina (18 – 82), a sudjelovalo je 68,9 posto bilo žena. Srednja vrijednost CAL-a za grupu dijabetičara iznosila je 3,44 milimetra, medijan je bio 3,08 milimetara, a standardna devijacija 1,45 milimetara.

Za HP-test identificirana su tri moguća genotipa u uzorku dobivenom od 119 brazilskih pacijenata. Na slici 1. vidi se da HP-test pokazuje jasnu distinkciju između tri genotipa koristeći se ljudskom DNK.

Results

The mean age of the patients was 45.5 (18 – 82) years, 68.9 % being female. The mean CAL of the diabetic patients group was 3.44mm, median 3.08mm and standard deviation of 1.45mm.

For the HP assay, the three possible genotypes were identified in a Brazilian sample of 119 patients; Figure 1 shows that the HP assays allows a clear discrimination of the three different genotypes, using human DNA.



Slika 1. A, B, C HP-test za detekciju -44 DEFB1 SNP; genotipovi C/C, C/G i G/G
Figure 1 A, B, C HP assay for detection of the -44 DEFB1 SNP. Genotypes C/C, C/G and G/G respectively, in the sample evaluated.

Tablica 1. Frekvencije genotipova gena DEFB1 u brazilskoj populaciji dijabetičara i zdravoj kontrolnoj skupini
Table 1 Genotypes frequencies in DEFB1 gene in a Brazilian diabetic population and healthy controls

Genotipovi • Genotypes	Pacijenti • Patients			P vrijednost • P value*
	Dijabetičari • Diabetic patients		Zdravi pacijenti • Healthy controls	
	Parodontitis • Periodontitis %	Bez parodontitisa • Without periodontitis %	%	
C / C	50.0 (22/44)	68.3 (28/41)	53.0 (18/34)	
C / G	34.1(15/44)	29.3 (12/41)	35.3 (12/34)	=0.232
G / G	15.9 (7/44)	2.4 (1/41)	11.7 (4/34)	

* Chi-Square test, interval značajnosti na 5 %, interval pouzdanosti na 95 % • Chi-Square test, significance level at 5%, confidence level at 95%

Tablica 2. Frekvencije alela -44 gena DEFB1 u brazilskoj populaciji dijabetičara i zdravoj kontrolnoj skupini
Table 2 Frequencies of the alleles -44 DEFB1 gene in a Brazilian diabetic population and healthy controls

Alele • Allele	Pacijenti • Patients		
	Dijabetičari • Diabetic		Zdravi pacijenti • Healthy Controls n (%)
	Parodontitis • Periodontitis n (%)	Bez parodontitisa • Without periodontitis n (%)	
C	67.0 (59/88)	83.0 (68/82)	70.6 (48/68)
G	33.0 (29/88)	17.0 (14/82)	29.4 (20/68)

Distribucija genotipova u uzorku pokazala je da 68 (57,1 %) ispitanika ima C/C, 39 (32,8 %) C/G i 12 (10,1 %) G/G. Nije bilo značajnih razlika između dijabetičara s parodontitisom ili bez njega i zdravih pacijenata (tablica 1.). Genotip homozigotnog mutanta bio je češći kod pacijenata s dijabetesom i parodontitisom.

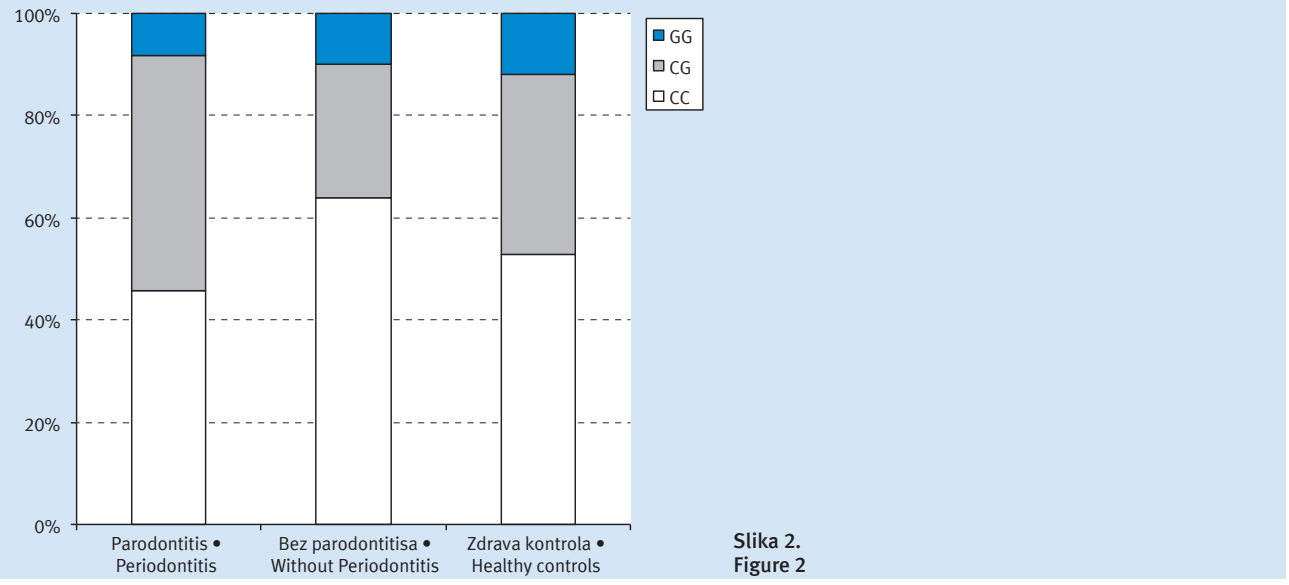
Frekvencija alela nalazi se u tablici 2. Mutirana G-alela češća je kod dijabetičara s parodontitisom. Uzimajući u obzir frekvencije alela, nije bilo značajnih razlika između grupa.

Ako uzimamo u obzir srednju vrijednost CAL-a kod dijabetičara i zdravih pacijenata u odnosu prema genotipovima, nema nikakve razlike (p=0,49). Između tri grupe viši postotak genotipa C/C pronađen je u grupi s dijabetičarima koji imaju parodontitis, te je postotak između homozigota (C/C) i heterozigota (C/G) vrlo sličan (Slika 2.).

Regarding the distribution of genotypes, the sample presented 68 (57.1%) subjects with C/C, 39 (32.8%) C/G and 12 (10.1%) G/G. There were no significant differences between diabetic with or without periodontitis and healthy controls (Table 1). The genotype homozygous mutant was more frequently found in diabetic patients with periodontitis.

The allele frequency is shown in Table 2. The allele mutant G was more frequently found in the diabetic periodontitis group. Considering allele frequencies there were no significant differences among the groups.

When considering the mean CAL in diabetic patients and healthy controls in relation to genotypes, no differences were established (p=0.49). Among the three groups, a higher percentage of the genotype C/C was found; in the group with diabetes and periodontitis the percentages between homozygous (C/C) and heterozygous (C/G) were similar (Figure 2).



Slika 2.
Figure 2

Rasprava

U ovom istraživanju korišten je HP-test kako bi se odredio polimorfizam na kodonu -44 gena DEFB1 na uzorku dijabetičara u brazilskoj populaciji u odnosu na uzorak zdrave populacije. Ova metoda omogućila je raspoznavanje tri moguća genotipa u uzorku. S pomoću krivulje pojačavanja mogao se identificirati genotip svakog pacijenta. Uzorci koji su pokazali raniji Ct s C HP-om smatrani su alelama C i homozigotnima, uzorci koji su imali raniji Ct s G HP-om smatrani su alelama G, a homozigotni i heterozigotni uzorci imali su sličan Ct u obje reakcije.

Tehnika je bila jednostavna i mnogi su istraživači izjavili su da su HP-om povećavali specifičnost i osjetljivost PCR-testa (Kabojev i suradnici, 2000.; Bonnioto i suradnici, 2004.). Drugi autori koristili su se različitim tehnikama, primjerice PCR-RFLP-om (Dörk, Stuhrmann, 1998.; Prado-Montes i suradnici, 2006. i 2009.) te PCR-om u stvarnom vremenu i sondiranjem (Prado-Montes i suradnici, 2009.) kako bi odredili SNP u genu DEFB1. Ovim metodama potrebna je

Discussion

In the present study the HP assay was used to evaluate the polymorphism in codon -44 DEFB1 gene in a sample of diabetic Brazilian adult population in comparison with healthy controls. This method allowed distinguishing the three possible genotypes in the sample. Through amplification curve it was possible to identify the genotype in each patient. Samples which showed an earlier *Ct* with C HP were considered allele C homozygous, samples that had an earlier *Ct* with G HP were considered allele G homozygous and heterozygous samples showed similar *Cts* in both reactions.

The technique was simple, feasible, and previous authors reported that HPs increase the specificity and the sensitivity of PCRs assay (Kaboev et al., 2000; Bonnioto et al., 2004). Other authors used different techniques such as PCR-RFLP (Dörk, Stuhrmann, 1998; Prado-Montes et al., 2006; Prado-Montes et al., 2009), real time PCR and probes (Prado-Montes et al., 2009) to evaluate the SNP in DEFB1 gene.

dodatna manipulacija nakon PCR-tehnike te su primeri korišteni u HP-testu daleko superiorniji od konvencionalnih linearnih primera za otkrivanje SNP-a, što je i prije bilo dokazano (Hazbon, Alland, 2004.).

β-defensini su porodica antimikrobnih peptida i sastavni su dio prirođene imunosti. Genetska varijacija u genu DEFB1 može uzrokovati promjene u ekspresiji i konfiguraciji peptida. Takve promjene katkad su povezana s odgovorom na patološko stanje, a mogu rezultirati i podložnošću ili otpornošću prema određenim tipovima infekcija (Jurevic i suradnici, 2002.). U ovom istraživanju procijenjena je povezanost između polimorfizma DEFB1 -44C/G u uzorku brazilske populacije dijabetičara s parodontitisom ili bez njega, u usporedbi sa zdravim pacijentima. Nisu uočene nikakve razlike između grupa. U zdravoj skupini zabilježen je veći postotak homozigotnog genotipa (C/C), a u skupini dijabetičara s parodontitisom veći postotak homozigotnog mutiranog gena (G/G) u usporedbi s ostalim grupama.

Genetska varijabilnost može uzrokovati razlike u frekvenciji genotipova i alela te promijeniti odgovor domaćina, ovisno o etnicitetu (Kocsis i suradnici, 2008), ali su Jurevic i njegovi kolege (2002.) pokazali da nema razlika u SNP-u na mjestu -44 DEFB1 gena u odnosu na etničku pripadnost.

Polimorfizam DEFB1 -44C/G povezan je s mnogo patoloških stanja, primjerice s kroničnom opstruktivnom bolesti pluća (Matsushita i suradnici, 2002.), kandidom u ustima (Jurevic i suradnici, 2003.), opasnosti od virusa ljudske imunodeficijencije tipa 1 u bijeloj populaciji (Braida i suradnici, 2004.), sepsom (Chen i suradnici, 2007.), Chronovom bolesti (Kocsis i suradnici, 2008.), atopičnim dermatitisom (Kim i suradnici, 2009.), lepromatoznom gubom (Prado-Montes i suradnici, 2009.) i dijabetesom (Guimarães i suradnici, 2009.). U rijetko kojem istraživanju bila je posvećena pozornost polimorfizmu gena DEFB1 i parodontitisu. Tako su Boniotto i suradnici (2004.) procijenili SNP gena DEFB1 i parodontitis koristeći se HP-testom, ali ovo istraživanje prvo je u kojemu je demonstrirano korištenje HP-testa kako bi se obavila tipizacija SNP-a kod dijabetičara s parodontitsom ili bez njega i usporedila s uzorkom zdrave populacije.

Uzimajući u obzir ukupan broj sudionika, rezultati ovog istraživanja, kad je riječ o genotipovima, podudaraju se s rezultatima istraživanja Boniotta i suradnika (2004.) ali ne slažu se s prijašnjim studijama (Braida i suradnici, 2004.; Milanese i suradnici, 2006.; Chen i suradnici, 2007.; Baroncelli i suradnici, 2009.; Guimarães ei suradnici, 2009.). Uzimajući u obzir frekvencije alela, ovo istraživanje pokazuje veći postotak G-alele negoli ostala istraživanja (Jurevic i suradnici, 2002. i 2003.; Boniotto i suradnici, 2004.; Braida i suradnici, 2004.; Milanese i suradnici, 2006.; Chen i suradnici, 2007.; Guimarães i suradnici., 2009.). Takvi rezultati mogli bi se pripisati etničkim razlikama koje mogu utjecati na distribuciju SNP-a, uzimajući u obzir da je istraživanje provedeno na sjeveroistočnoj populaciji Brazilaca. Na istoj populaciji provedeno je nekoliko istraživanja, kako bi se procijenio utjecaj genetskih čimbenika u nastanku parodontnih bolesti (Araújo i suradnici, 2009.). U svojem istraživanju su Jurevic i suradnici (2003.) pokušali dokazati da je G-alela povezana s protektivnim učinkom kod pacijenata s dijabetesom kad je

In these methods manipulation after the PCR run is needed, and the superiority of the primers used in HP assay, over conventional linear primers for SNP detection, has previously been demonstrated (Hazbon, Alland, 2004).

The β-defensins are a family of antimicrobial peptides that have been implicated in the innate host defence system. Genetic variation in genes DEFB1 may underline changes in expression or configuration of the peptide. Such changes may be associated with alteration in innate response to pathologic challenge and may also result in susceptibility or resistance to certain types of infections (Jurevic et al., 2002). The association of DEFB1 -44C/G polymorphism in a Brazilian diabetic population with or without periodontitis in comparison to healthy control patients was evaluated in the study and no differences were observed among groups. The healthy control group exhibited a higher percentage of genotype homozygous wild (C/C), while the diabetic periodontitis group exhibited higher percentages of homozygous mutant (G/G) when compared to the other groups.

The genetic variability may cause differences in frequency of genotypes, alleles and, consequently, changes in host response according to ethnicity (Kocsis et al., 2008), but Jurevic et al. (2002) have shown no differences considering ethnics in relation to SNP in site -44 of DEFB1 gene.

DEFB1 -44C/G polymorphism has been associated with many pathologic conditions such as chronic obstructive pulmonary disease (Matsushita et al., 2002), *Candida* carriage in the mouth (Jurevic et al., 2003), risk of human immunodeficiency virus type 1 in the Caucasian population (Braida et al., 2004), sepsis (Chen et al., 2007), Crohn´s disease (Kocsis et al., 2008), atopic dermatitis (Kim et al. 2009), lepromatous leprosy (Prado-Montes et al., 2009) and diabetes (Guimarães et al., 2009). A small number of studies have been made targeting DEFB1 polymorphism considering periodontal conditions, such as Boniotto et al. (2004) that evaluated SNP of DEFB1 gene and periodontitis using HP assay. The present study is the first report of the application of a HP assay to SNP genotyping in a diabetic population with or without periodontitis and healthy controls.

Considering the total patient sample, the results of the present research, regarding genotypes, are in agreement with Boniotto et al., (2004) but it disagrees with some previous researches (Braida et al., 2004; Milanese et al., 2006; Chen et al., 2007; Baroncelli et al, 2009; Guimarães et al., 2009). In relation to alleles frequencies this research shows a higher percentage of G allele than others (Jurevic et al., 2002; Jurevic et al. 2003; Boniotto et al., 2004; Braida et al., 2004; Milanese et al., 2006; Chen et al., 2007; Guimarães et al., 2009). This finding could be explained by ethnic differences that may influence the frequency and distribution of the SNP considering the sample was constituted by a Northeast Brazilian population, and a few studies have been made in such population to evaluate genetic factors interference in periodontal disease (Araújo et al., 2009). In a previous study, Jurevic et al. (2003) tried to demonstrate that G allele was associated with protective effect in diabetic patients in relation to *Candida* carriage, but no significant differences were found between diabetic and non-diabetic populations. The

riječ o prisutnosti kandide, ali nisu pronašli nikakvu statistički značajnu razliku između ispitanika s dijabetesom i bez njega. U grupi pacijenata s dijabetesom i parodontitisom zabilježena je najveća frekvencija mutacije genotipa i alela.

Nedostatak statistički značajnih razlika može se objasniti činjenicom da ljudski beta-defensin 1 ne djeluje na bakterije koje uzrokuju parodontitis i ne potiče nastanak parodontne bolesti. U prijašnjim istraživanjima dokazano je da treponeme služe kao fizička prepreka između peptida nastalih u epitelnim stanicama i ostalih bakterija osjetljivih na defensine koje potiču nastanak parodontitisa, što uvelike pridonosi preživljavanju tih bakterija u gingivalnom sulkusu (Brissete i suradnici, 2004.; Brissete, Lukehart, 2007.). *Treponema denticola* otporna je i na peptid β-defensin-1 (Brissete, Lukehart, 2002.). Unatoč svemu, nema podatka koji bi upućivali na biološku aktivnost peptida na *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia*, viruse *i* ostale mikroorganizme odgovorne za patogenezu parodontne bolesti.

Zaključak

Na kraju je procijenjena prisutnost SNP DEFB1 kod dijabetičara s parodontitisom ili bez njega, u usporedbi sa zdravom kontrolnom skupinom. Ovo istraživanje prvo je u kojemu se analizirala moguća povezanost između DEFB1 kod dijabetičara i stanja parodonta. Nije bilo statistički značajnih razlika te bi trebalo obaviti daljnja istraživanja kako bismo rasvijetlili ulogu polimorfizma DEFB1 u odnosu na parodontitis, odnosno biološki utjecaj DEFB1 na mikroorganizme odgovorne za nastanak parodontitisa.

diabetic periodontitis group exhibited the most frequencies of genotype and allele mutant.

The lack of significant differences may be explained by the fact that human beta-defensin 1 is not active against periodontal bacteria and does not play a role in periodontal disease. Previous studies demonstrated that Treponemas may serve as a physical barrier protection between epithelium derived antimicrobial peptides and other defensins-sensitive bacteria involved in periodontal disease, contributing to the survival of these and other bacteria in the gingival crevice (Brissete et al., 2004; Brissete, Lukehart, 2007). And also that *Treponema denticola* is resistant to peptide β-defensin-1 (Brissete, Lukehart, 2002). Nevertheless, no data are available regarding the biological activity of this peptide against *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, viruses* and other microorganisms implicated in the pathogenesis of the periodontal disease.

Conclusion

in conclusion, the presence of SNP DEFB1 was evaluated in diabetic patients with or without periodontitis in comparison to healthy controls, being the first report investigating a possible association between DEFB1 in diabetic patients in relation to their periodontal condition. No significant differences were found and further studies should be performed in order to elucidate the role of DEFB1 polymorphisms in relation to periodontal disease, mainly its biological activity against the microorganisms involved in periodontitis.

Abstract

Aim: Defensins are cationic antimicrobial peptides expressed in epithelial cells. Such peptides exhibit antibacterial, antifungal and antiviral properties, and are a component of the innate immune response. It has been suggested that they have a protective role in the oral cavity. This study evaluated the DEFB1 polymorphism in diabetic patients with or without periodontitis in comparison to healthy controls. **Material and Methods:** We used Hairpin-Shaped Primer (HP) assay to study the distribution of the -44 C/G SNP (rs1800972) in 119 human DNAs obtained from diabetic patients and healthy control patients. **Results:** The results indicate that there are no differences in distribution between groups and that in diabetic periodontitis patients the homozygous mutant could be found more frequently. **Conclusion:** Further studies are necessary in order to investigate the role of DEFB1 polymorphisms in diabetic periodontitis patients and the influence of the peptide in periodontal pathogens.

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Key words
Defensins; Antimicrobial Cationic Peptides; DEFB1 protein, human; Epithelial Cells; Diabetes Complications; Periodontitis

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