

Impact of *DEFB1* gene regulatory polymorphisms on hBD-1 salivary concentration

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ABSTRACT

Objectives: Human β -defensin 1 (hBD-1) is an antimicrobial peptide involved in epithelial defence of various tissues, also present in the saliva. Individual genetic variations within the *DEFB1* gene, encoding for hBD-1, could influence gene expression and protein production.

Design: Three *DEFB1* polymorphisms at 5' untranslated region (UTR), $-52G > A$ (rs1799946), $-44C > G$ (rs1800972) and $-20G > A$ (rs11362), and two polymorphisms at *DEFB1* 3' UTR, $c^*5G > A$ (rs1047031) and $c^*87A > G$ (rs1800971), were analysed by direct sequencing and correlated with hBD-1 salivary concentration (tested with enzyme-linked immunosorbent assay (ELISA)) in 40 healthy subjects.

Results: Significant associations were found between individuals presenting different *DEFB1* polymorphisms at positions -52 and -44 of the gene and hBD-1 salivary concentrations: $-52 G/G$ carriers had higher levels of protein than G/A and A/A ; $-44C/G$ subjects showed a higher protein concentration than homozygous wild-type C/C . For the $-20G > A$, $c^*5G > A$ and $c^*87A > G$ polymorphisms, no statistically significant differences were found. Combined haplotype analysis confirmed the results obtained considering the single-nucleotide polymorphisms (SNPs) singularly.

Conclusion: Polymorphisms in the *DEFB1* gene influence hBD-1 production and, therefore, could modify the innate immune system responses and, consequently, the oral health.

1. Introduction

β -Defensins are small cationic antimicrobial peptides that play an important role in the innate immune system, recognizing and protecting individuals from pathogens without prior exposure.¹ Among the different types of β -defensins identified

so far,²⁻⁶ human β -defensin 1 (hBD-1) is an important antimicrobial peptide acting in epithelial defence against infections^{7,8}; it has been detected on the mucosal surface of airways, gastrointestinal tract, urogenital tissue,⁹ oral cavity⁶ and gingival tissues,⁸ and it has also been found in saliva.¹⁰

Saliva has a mechanical cleaning activity in healthy individuals, and it preserves the oral cavity from different

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biological, chemical and mechanical injuries by rinsing and delivering antimicrobial peptides and proteins such as hBD-1¹¹: this molecule is present in saliva,¹⁰ and it may support the general oral health. In fact, low levels of hBD-1 could influence disease susceptibility or the response of the immunity system during infection.¹² hBD-1 is constitutively produced, but its expression could also be induced by inflammatory or microbial stimuli⁸ and it could be also influenced by genetic variants within the *DEFB1* gene (8p22-23) encoding for hBD-1 peptide. In this study, three *DEFB1* polymorphisms at 5' untranslated region (UTR), -52G > A (rs1799946), -44C > G (rs1800972) and -20G > A (rs11362), and two *DEFB1* polymorphisms at 3' UTR, c*5G > A (rs1047031) and c*87A > G (rs1800971), were analysed in relation with the hBD-1 level.

Few previous studies have analysed the role of regulatory 5' UTR *DEFB1* polymorphisms (-52G > A, -44C > G and -20G > A) in determining hBD-1 expression,^{6,13-17} and the results were sometimes discordant.^{14,15} Instead, for two 3' UTR *DEFB1* polymorphisms (c*5G > A and c*87A > G), no previous study was conducted for determining their influence on gene expression. These latter two polymorphisms are localized in possible microRNA (miRNA)-binding sites, so they are potential modifiers of hBD-1 protein expression.⁸ The aim of the present study is to clarify the possible functional role of the above-mentioned five *DEFB1* polymorphisms on hBD-1 expression *in vivo*.

2. Materials and methods

2.1. Patient population

Forty healthy Italian individuals (22 women and 18 men, median age = 27.4 years, range = 21-39) were recruited at the Division of Oral Medicine and Pathology (Dental Science Department, University of Trieste, Trieste, Italy). From each patient who signed written informed consent, a non-stimulated collection of saliva and a blood sample were obtained.

Professional nurses performed the blood sampling using Vacuette[®] blood collection tubes (Greiner Bio One GmbH, Frickenhausen, Germany); the tubes were immediately stored, for no more than 24 h, at 4 °C and then carried to the laboratories of the Institute for Maternal and Child Health (IRCCS 'Burlo Garofolo', Trieste, Italy).

Each patient was asked to spit for 3 min into a plastic biologic container; then, the collected saliva was withdrawn and released into an Eppendorf tube (Eppendorf, Hamburg, Germany). Each sample was centrifuged for 10 min at 10,000 rpm in a micro-centrifuge (Sanyo[®] MSE Micro Centaur, MSE, London, UK). All samples were immediately stored at a temperature of -20 °C and carried to the laboratories of the Institute for Maternal and Child Health (IRCCS 'Burlo Garofolo', Trieste, Italy).

All study experiments and procedures were performed in accordance with the ethical standards of the Declaration of Helsinki, and the Bio-Ethical Committee of IRCCS Burlo Garofolo approved the study (RC03/04, L1055, protocol number 118/10).

2.2. *DEFB1* genotyping

Genomic DNAs were extracted from whole blood using the automatic DNA extractor NucliSENS[®] easyMAG[®] (bioMérieux, Marcy l'Etoile, France), and samples were genotyped for *DEFB1* -52G > A (rs1799946), -44C > G (rs1800972), -20G > A (rs11362), c*5G > A (rs1047031) and c*87A > G (rs1800971) polymorphisms by direct sequencing.

Using PCR buffer 1×, one unit of Taq Gold, 0.2 mM deoxynucleotide triphosphates (dNTPs) and 2 mM MgCl₂, polymerase chain reactions (PCRs) were carried out in a Gene-Amp 9700 Thermal Cycler (Applied Biosystems—Life Technologies, Foster City, CA, USA) using the following primers: forward 5' GTGCCATGTGACTGCTGACT 3' and reverse 5' AGCCATCGGAGACTCACATC 3' for 5' UTR polymorphisms and forward 5' ACTCTCCGGTGTTTTGCA GT 3' and reverse 5' ACCTGTCTCACGTTCCAACC 3' for 3' UTR polymorphisms. The cycling was performed with an initial denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 20 s, at the annealing temperature of 55 °C for 30 s and 72 °C for 30 s with a final extension to 72 °C for 7 min. The successful DNA amplification and the absence of non-specific reaction products were verified using electrophoresis run of the amplicons on 2% agarose gel. DNA sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 2.0 (Applied Biosystems). DNA sequences were run on an automated ABI Prism 3100 Genetic Analyser using the 3130 Data Collection Software (Applied Biosystems); sequences were handled using the 4Peaks software (available at <http://nucleobytes.com/index.php/4peaks>).

2.3. hBD-1 salivary concentration

The collected salivary samples were diluted 1:25 in water, and the hBD-1 salivary concentration was measured with a human β-defensin 1 ELISA (enzyme-linked immunosorbent assay) kit (Cat. No. 100-240-BD1, Alpha Diagnostic, San Antonio, TX, USA) according to the manufacturer's instructions. Each assay sample was conducted in duplicate. The absorbance was measured using a GloMax[®]-Multi Detection System (Promega, Fitchburg, WI, USA).

2.4. Statistical analysis

DEFB1 polymorphisms and allele and genotype frequencies were calculated by direct counting, while haplotype frequencies and linkage disequilibrium were computed using the Arlequin software (version 3.1).¹⁸

The Kruskal-Wallis test for unpaired samples and the two-sided Kolomogorov-Smirnov test were used for comparing the salivary hBD-1 concentration (obtained with ELISA assay) between the different *DEFB1* genotypes and haplotypes.

All the statistical analyses were carried out using the open-source R package version 3.1.0.¹⁹

3. Results

Five polymorphisms in the *DEFB1* gene were analysed: -52G > A, -44C > G and -20G > A in the 5' UTR of the gene

and c*5G > A and c*87A > G in the 3' UTR. All single-nucleotide polymorphism (SNP) frequencies were in Hardy-Weinberg equilibrium.

The concentration of hBD-1 in the saliva of all individuals tested showed a range varying from 0.60 to 21.99 ng/ml; the median value was 5.01 ng/ml. The three possible genotypes at position -52 (G/G, A/A and G/A) were associated with different hBD-1 production (Kruskal-Wallis test, $p = 0.01$); homozygous wild-type subjects G/G had a higher hBD-1 concentration than homozygous mutated A/A (median 6.89 and 2.87 ng/ml, respectively; Kolomogorov-Smirnov test, $p = 0.01$), while heterozygous G/A presented intermediate concentrations (5.01 ng/ml).

A statistically significant difference of hBD-1 levels was also found between individuals carrying the -44C > G C/G and C/C genotypes: heterozygous subjects showed a higher protein concentration in the saliva than the homozygous wild type (6.89 and 4.69 ng/ml, respectively; Kruskal-Wallis test, $p = 0.04$). Only one subject was homozygous G/G, so it was not included in the analysis.

For the -20G > A, c*5G > A and c*87A > G polymorphisms, no statistically significant differences in median hBD-1 concentrations related to DEFB1 genotypes were found (Table 1).

The five DEFB1 polymorphisms combined in our samples to form five major combined haplotypes (ACGGA/ACGGA, ACGGA/GCAAA, ACGGA/GCAGA, GCAGA/GCAGA and GCAGA/GGGA) and other minor haplotypes (frequency < 0.05, data not shown).

To examine if the effect of the DEFB1 -52G > A and -44C > G SNPs was also reflected at the haplotype level, statistical tests were applied considering the presence or

absence of the ACxxx haplotype, which combined the two major alleles -52A and -44C (and where x indicates any allele for -20G > A, c*5G > A and c*87A > G SNPs), both associated with lower hBD-1 levels when singularly considered. A significant difference in hBD-1 protein expression was obtained comparing subjects carrying the ACxxx/ACxxx combined haplotype and subjects not carrying AC (Kolomogorov-Smirnov test, $p = 0.001$), confirming results obtained considering each single SNP: individuals with ACxxx/ACxxx haplotypes had low protein concentration (median 2.87 ng/ml and mean 3.67 ng/ml), while individuals with only one copy of the ACxxx haplotype presented an intermediate concentration (median 5.01 ng/ml and mean 7.11 ng/ml) and subjects with no ACxxx haplotypes (i.e., GCxxx or GGxxx) showed higher protein concentration (median 6.88 ng/ml and mean 8.23 ng/ml).

4. Discussion

In this functional study, we investigated the possible association between DEFB1 polymorphisms and in vivo salivary levels of hBD-1 in an Italian population of 40 healthy individuals, finding a genotype-related different expression associated with two of these polymorphisms, namely -52G > A and -44C > G.

In fact, a higher hBD-1 concentration was found in subjects carrying the -52G > A G/G (median 6.89 ng/ml) compared to -52G/A and -52A/A (5.01 and 2.87 ng/ml, respectively) as well as in -44C > G C/G subjects compared to C/C (6.89 and 4.69 ng/ml, respectively). Additionally, haplotype analysis showed a statistically significant difference in protein concentration

Table 1 – Analysis of hBD-1 salivary concentration according to DEFB1 genotypes.

DEFB1	Subjects n = 40	hBD-1 salivary concentration		Chi-square Degree of freedom	p-Value
		Average (ng/ml)	Median (ng/ml)		
-52G > A rs1799946					
G/G	11 (0.28)	8.23	6.89	$\chi^2 = 8.6$; df = 2	$p = 0.01^*$
G/A	20 (0.50)	7.11	5.01		
A/A	9 (0.22)	3.67	2.87		
-44C > G rs1800972					
C/C	32 (0.80)	6.01	4.69	$\chi^2 = 4.2$; df = 1	$p = 0.04^*$
C/G	7 (0.18)	9.05	6.89		
G/G	1 (0.02)	9.94	NA		
-20G > A rs11362					
G/G	14 (0.35)	5.60	4.65	$\chi^2 = 2.4$; df = 2	$p = 0.29$
G/A	20 (0.50)	6.99	5.62		
A/A	6 (0.15)	7.91	5.95		
c*5G > A rs1047031					
G/G	28 (0.70)	6.02	5.24	$\chi^2 = 0.5$; df = 1	$p = 0.50$
G/A	11 (0.28)	8.41	4.85		
A/A	1 (0.02)	4.81	NA		
c*87A > G rs1800971					
A/A	38 (0.95)	6.75	5.24	$\chi^2 = 0.2$; df = 1	$p = 0.62$
G/A	2 (0.05)	4.71	4.71		
G/G	0 (0.00)	NA	NA		

* Statistically significant p-value.

between subjects carrying ACxxx/ACxxx (–52A and –44C alleles in homozygosis) and GCxxx/GCxxx (or GCxxx/GGxxx) combined haplotypes confirming the analysis conducted for single polymorphisms, and suggesting that the two polymorphisms may act synergistically to modulate hBD-1 levels: the ACxxx haplotype was associated with low protein concentration compared to the GCxxx or GGxxx haplotype (median 2.87 vs. 6.89 ng/ml).

To the best of our knowledge, very few studies have investigated the possible association between these *DEFB1* polymorphisms and hBD-1 protein expression. Ikuta et al.²⁰ studied the association between *DEFB1* SNPs and hBD-1 concentration in gingival crevicular fluid using an ELISA assay. Although the number of samples was small, the authors observed that the hBD-1 concentration was significantly lower in subjects with the –44 CC genotype than in those with other genotypes, in accordance with our results obtained in saliva.

Unlike our study, James et al.²¹ observed higher median serum hBD-1 concentration in –52G > A A/A homozygotes and G/A heterozygotes compared to G/G, and also lower median serum hBD-1 level in c*5G > A A/A homozygotes compared to G/A or G/G. In our study, no differences were seen in hBD-1 levels according to c*5G > A SNPs, and only one individual was c*5G > A A/A homozygous. The allele and genotype frequencies of the SNPs were not reported in the study by James et al., which moreover was performed in serum samples, not in saliva, and in pregnant women, so a direct comparison with our study cannot be performed.

A few works have also considered *DEFB1* polymorphisms in gene expression. Kalus and co-workers¹³ reported a higher constitutive *DEFB1* messenger RNA (mRNA) expression and a more efficient antimicrobial activity in –44G/G human oral keratinocytes cultured in vitro compared to –44C/C; Kalus et al. also observed an increased *DEFB1* mRNA expression in COS-7 cells transfected with reporter construct for the GGG haplotype compared to GCA, GCG and ACG constructs. Although the study by Kalus et al. considered the *DEFB1* mRNA while the present study analysed hBD-1 expression in saliva, the findings of the two studies are similar: in fact, the –44G allele and ACG haplotypes were associated, respectively, with a higher and reduced *DEFB1* expression in the study by Kalus et al., and with a higher and reduced hBD-1 expression in the present study.

An increased luciferase production in the presence of constructs with the –44G allele in DU145 or TSU-Pr1 cell lines was also reported by Sun et al.,¹⁴ while Milanese and co-workers¹⁵ observed a reduction in luciferase expression with the –44G plasmid in the Caco-2 cell line, as well as in the presence of the –52A allele (although the latter not in a statistically significant way). Kalus et al.¹³ suggested that these differences could be determined by a post-transcriptional regulation of the protein via the RNA-binding domain²² or by the different mRNA stabilities in various cell types,¹³ in fact the DU145 cell line derived from human prostate cancer cell lines, TSU-PR1 from bladder cancer cells and the Caco-2 cells from human epithelial colorectal adenocarcinoma cells. Nevertheless, the studies by both Milanese et al.¹⁵ and Sun et al.¹⁴ were conducted with an in vitro model system and they tested mRNA expression, while in our study we considered

mature protein level and hBD-1 expression ‘in vivo’ on salivary samples from healthy individuals.

Another study by Nurjadi et al.¹⁶ analysed the influence of *DEFB1* polymorphisms in gene expression: they observed that the –52A/A, –44G/G and –20G/G genotypes were associated with higher *DEFB1* mRNA expression in healthy and wounded skin, and the –52/–44/–20 GGG haplotype presented higher protein expression than ACG and GCA (assuming that hBD-1 expression followed GGG > ACG > GCA). The findings of Nurjadi et al. are similar to ours for the –44C > G polymorphism, but not for the other two polymorphisms, as in our study the –52A > G A/A genotype was associated with lower protein expression and no significant association was found for the –20C > G polymorphism. The possible explanation of this different finding could be due to the different materials analysed: in the present study, we examined hBD-1 in saliva (secretion of oral mucosa) while Nurjadi et al. analysed hBD-1 in skin epithelium, and not in wound secretion or pus. Additionally, Nurjadi et al. studied the effect of only one haplotype on the hBD-1 expression; instead, in our study we considered the haplotype combination presented in each individual.

In conclusion, in this work, we reported the association between *DEFB1* genetic variants and hBD-1 in vivo protein levels, showing that these polymorphisms could be effectively involved in the regulation of protein production in saliva. Further studies are forecasted to verify whether *DEFB1* genetic variants are associated with an increased susceptibility of the oral mucosa to infectious/chemical damages or with specific oral diseases.

Conflict of interest statement

None of the authors has any potential financial conflict of interest related to this manuscript.

Authors' contribution

VP performed *DEFB1* genotyping and hBD-1 ELISA experiments and drafted the manuscript; LZ performed the statistical analyses and participated in writing the manuscript; RDL contributed to the supervision of the clinical protocol; MB was responsible for the setting of the clinical protocol and management of patients; GO participated in sample collection and manuscript revision; LC and MG collected and prepared the samples; GA provided technical support and extracted the DNAs; GP and SC critically revised the manuscript; and LS conceived the study and supervised the experiments and the analysis of the results.

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Competing interests

None declared.

Ethical approval

All study experiments and procedures have been performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the IRCCS Burlo Garofolo Ethical Committee [RC03/04, L1055, protocol number 118/10].

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