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DEFB1 polymorphisms and salivary hBD-1 concentration in Oral Lichen Planus patients and healthy subjects

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ABSTRACT

Objectives: The aetiology of Oral Lichen Planus (OLP), a chronic inflammatory disease of oral mucosa, is not yet well understood. Since innate immunity may be hypothesized as involved in the susceptibility to OLP, we studied human beta defensin 1 (hBD-1) an antimicrobial peptide constitutively expressed in the saliva, looking at functional genetic variants possibly able to diminish hBD-1 production an consequently conferring major susceptibility to OLP.
Design: We analysed three *DEFB1* polymorphisms at 5′ UTR, −52G > A (rs1799946), −44C > G (rs1800972), −20G > A (rs11362) and two *DEFB1* polymorphisms at 3′UTR, c*5G > A (rs1047031), c*87A > G (rs1800971), with the aim of correlating these genetic variants and hBD-1 salivary level in a group of OLP patients and in healthy subjects. We also evaluated hBD-1 salivary concentrations, using ELISA, in OLP and healthy controls.
Results: We compared hBD-1 concentrations in OLP and healthy subjects: hBD-1 concentration was significantly higher in OLP patients respect to control.
When considering the correlation between *DEFB1* polymorphisms genotypes and hBD-1 expression levels, significant results were obtained for SNPs −52G > A (p = 0.03 both in OLP patients and healthy individuals) and −44C > G (p = 0.02 in OLP patients).
Conclusions: hBD-1 production was different between OLP and healthy subjects (not age-matched with OLP). *DEFB1* gene polymorphisms, −52G > A and −44C > G, correlated with hBD-1 salivary concentrations.

1. Introduction

Oral Lichen Planus (OLP) is a chronic inflammatory disease of oral mucosa with a not well-defined aetiology (De Rossi & Ciarrocca, 2014; Wang & van der Waal, 2015). Current data suggest that the pathogenesis is closely related to an altered immune regulation mediated by cytotoxic T lymphocytes; T cells trigger apoptosis of oral epithelial keratinocytes causing lesions that are difficult to palliate (Eisen, Carrozzo, Bagan Sebastian, & Thongprasom, 2005; Porter, Kirby, Olsen, & Barrett, 1997). OLP affects adults aged 40–70 years, mainly women rather than men (F:M ratio 3:2) (Mazzarella, Femiano, Gombos, De Rosa, & Giuliano, 2006; Sugerman & Savage, 2002). Innate immunity may have a key role in the development of the pathology (Adami et al., 2014); since in the saliva antimicrobial peptides including defensins are present (Abiko, Nishimura, & Kaku, 2003), we can hypothesize their involvement in the development of OLP. Defensins mediate the first line of host defence and have been reported as implicated in maintaining the general oral health (Abiko et al., 2003), but the specific functions are yet unclear. Dysregulation in the production of these antimicrobial peptides might be involved in the development of oral pathologies, among them OLP. In particular, the beta defensin-1 (hBD-1) peptide is constitutively produced by oral epithelial cells (Dunsche et al., 2001; Krisanaprakornkit et al., 2000; Mathews et al., 1999) and gingival tissue (Krisanaprakornkit, Weinberg, Perez, & Dale, 1998), however it has been demonstrated that its expression could be modulated by inflammation

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(Abiko & Saitoh, 2007) and the highest hBD-1 concentration was observed in the inflamed epithelia (Dale & Fredericks, 2005; Krisanaprakornkit et al., 1998, 2000).

The gene encoding hBD-1 protein, *DEFB1*, (GeneID: 1672; www.ncbi.nlm.nih.gov/gene) spans over 7448 bp at locus 8p23.2-p23.1, and occurrence of genetic polymorphisms within *DEFB1* has been reported to possibly modulate the expression of the protein (Sahasrabudhe, Kimball, Morton, Weinberg, & Dale, 2000); our research group recently described significant association between *DEFB1* 5′ Untranslated Region (UTR) single nucleotide polymorphisms (SNPs) at position −52 and −44 and hBD-1 salivary concentrations in 40 healthy subjects (Polesello et al., 2015). Other works previously reported that promoter polymorphisms might alter transcriptional activity of *DEFB1* gene (Milanese, Segat, & Crovella, 2007; Sun et al., 2006).

In this study, three *DEFB1* polymorphisms at 5′ UTR, −52G > A (rs1799946), −44C > G (rs1800972), −20G > A (rs11362) and two *DEFB1* polymorphisms at 3′UTR, c*5G > A (rs1047031), c*87A > G (rs1800971) were analysed in relation with hBD-1 salivary level in a group of OLP patients and in healthy subjects with the aim of investigating if these functional *DEFB1* polymorphisms may influence the protein concentrations in these groups of subjects.

2. Materials and methods

2.1. Patient population

58 OLP patients (41 women and 17 men, median age = 65.04, range = 37-89 years) and 58 healthy Italian individuals (38 women and 20 men, median age = 35.03 years, range = 22–60 years) were recruited at the Division of Oral Medicine and Pathology (Dental Science Department, University of Trieste, Trieste, Italy); the patients were not in the clinic for dental work but for the regular follow up regarding OLP.

For the patients inclusion criteria were: age between 18 and 90 years and OLP diagnosis after oral incisional biopsy (study group), oral swab negative for yeast and bacterial infections; meanwhile exclusion criteria were: smokers, diabete mellitus, topical therapy (mouthwash, gel, toothpaste) and local and/or systemic steroid therapy. Subjects with healthy mucosa were visited before their enrollment in the present study and those affected by dental caries were excluded from the analysis.

For each subject saliva and blood samples were collected after obtaining a written informed consents from all subjects. From each patient, fasting for 2 h, the collection of unstimulated saliva spit for 3 min into a plastic biologic container, was obtained. 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 (Sanyo1 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). The blood sampling was performed by professional nurses using Vacuette1 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).

All experimental procedures have been performed in accordance with the ethical standards of the Declaration of Helsinki and

Table 1
DEFB1 polymorphisms genotype frequencies (and counts) and analysis of hBD-1 salivary concentrations according to *DEFB1* genotypes in OLP patients and healthy subjects. Hardy Weinberg (HWE) χ^2 and p-value are also reported.

	OLP (n° = 58)			Healthy subjects (n° = 58)		
SNPs	Genotypes	Median HBD-1 concentration (ng/ml)	p-value (Kruskal-Wallis test)	Genotypes	Median HBD-1 concentration (ng/ml)	p-value (Kruskal-Wallis test)
−52G > A rs1799946	G/G 25 (0.43) A/G 23 (0.39) A/A 10 (0.17) HWE χ^2 = 1.30; p = 0.25	G/G 11.43 A/G 11.66 A/A 8.41	χ^2 = 7.29, df = 2, p-value = 0.03*	G/G 21 (0.36) A/G 26 (0.45) A/A 11 (0.19) HWE χ^2 = 0.33; p = 0.56	G/G 7.05 A/G 5.01 A/A 3.61	χ^2 = 7.29, df = 2, p-value = 0.03*
−44C > G rs1800972	C/C 40 (0.69) C/G 16 (0.28) G/G 2 (0.03) HWE χ^2 = 0.06; p = 0.80	C/C 9.41 C/G 19.06 G/G 19.88	χ^2 = 7.83, df = 2, p-value = 0.02*	C/C 41 (0.71) C/G 12 (0.21) G/G 5 (0.09) HWE χ^2 = 6.20; p = 0.01	C/C 5.18 C/G 6.90 G/G 8.32	χ^2 = 3.32, df = 2, p-value = 0.19
−20G > A rs11362	G/G 19 (0.33) A/G 25 (0.43) A/A 14 (0.24) HWE χ^2 = 1.00; p = 0.32	G/G 10.43 A/G 13.59 A/A 7.07	χ^2 = 2.94, df = 2, p-value = 0.23	G/G 21 (0.36) A/G 28 (0.48) A/A 9 (0.15) HWE χ^2 = 0.004, p = 0.95	G/G 4.67 A/G 5.87 A/A 6.93	χ^2 = 2.63, df = 2, p-value = 0.27
c*5G > A rs1047031	G/G 40 (0.69) A/G 16 (0.28) A/A 2 (0.03) HWE χ^2 = 0.06; p = 0.80	G/G 11.90 A/G 8.69 A/A 6.99	χ^2 = 2.04, df = 2, p-value = 0.36	G/G 38 (0.65) A/G 18 (0.31) A/A 2 (0.03) HWE χ^2 = 0.005; p = 0.94	G/G 5.35 A/G 7.51 A/A 3.44	χ^2 = 3.61, df = 2, p-value = 0.16
c*87A > G rs1800971	A/A 56 (0.97) A/G 2 (0.03) G/G 0 (0.00) HWE χ^2 = 0.02; p = 0.89	A/A 11.34 A/G 6.08	χ^2 = 1.98, df = 1, p-value = 0.16	A/A 56 (0.97) A/G 2 (0.03) G/G 0 (0.00) HWE χ^2 = 0.02; p = 0.89	A/A 5.82 A/G 4.80	χ^2 = 0.30, df = 1, p-value = 0.59

*Statistically significant p-value.

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 peripheral whole blood with the automatic DNA extractor NucliSENS® easyMAG® (bioMérieux, Marcy l'Etoile, France). The samples were genotyped by direct sequencing for five *DEFB1* polymorphisms, 3 at 5′UTR −52G > A (rs1799946), −44C > G (rs1800972), −20G > A (rs11362), and 2 at 3′UTR c*5G > A (rs1047031) and c*87A > G (rs1800971).

The following primers, forward 5′ GTG CCA TGT GAC TGC TGA CT 3′ and reverse 5′ AGC CAT CCG AGA CTC ACA TC 3′ for 5′UTR polymorphisms and forward 5′ ACT CTC CGG TGT TTT GCA GT 3′ and reverse 5′ ACC TGT CTC ACG TTC CAA CC 3′ for 3′UTR polymorphisms were used for polymerase chain reactions, carried out in Gene-Amp 9700 Thermal cycler (Applied Biosystems – Life Technologies, Foster City, California, USA) using PCR buffer 1×, 1 unit of Taq Gold, 0.2 mM dNTPs and 2 mM MgCl2. The cycling was performed with an initial denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 20s, 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. Electrophoresis run (2% agarose gel) was performed to check the successful DNA amplification and the absence of non-specific reaction products. PCR products were sequenced 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 Biosystem); the sequences were analyzed using the 4Peaks software (available at http://nucleobytes.com/index.php/4peaks site).

2.3. HBD-1 salivary concentration

Saliva samples were obtained from spit, centrifuged for 10 min at 10000 rpm (Sanyo MSE Micro Centaur, MSE, London, United Kingdom) and stored at −20 °C before analysis.

At the time of the test, the specimens were diluted 1:25 in water and hBD-1 salivary concentration was measured with Human Beta Defensin 1 ELISA kit, (Cat. No. 100–240-BD1, Alpha Diagnostic, San Antonio, TX, USA) using manufacturer's instructions. The absorbance was measured with GloMax®-Multi Detection System (Promega, Fitchburg, WI, USA).

2.4. Statistical analysis

DEFB1 polymorphisms, allele and genotype frequencies were calculated by direct counting, while haplotype frequencies and linkage disequilibrium were computed using the Arlequin software (version 3.1) (Excoffier & Lischer, 2010).

Correlations between *DEFB1* genotypes (and haplotypes) and salivary hBD-1 concentration were analysed using Kruskall Wallis test for unpaired samples and two-sided Kolmogorov Smirnov test.

All the statistical analyses were carried out using the open-source R package version 3.1.0 (RcoreTeam, 2015).

3. Results

Salivary hBD-1 concentration varied in a range of 1.57–30.48 ng/ml, median = 11.11 ng/ml for OLP patients and of 0.60–21.99 ng/ml, median = 5.64 ng/ml for healthy subjects. Statistically significant result was obtained by comparing hBD-1 concentrations between OLP and healthy subjects (Kolmogorov-Smirnov test, p-value = 0.00018, data not shown).

For the five *DEFB1* polymorphisms analysed in this study, −52G > A, −44C > G and −20G > A localized at 5′ UTR region and c*5G > A and c*87A > G at the 3′ UTR region, SNPs frequencies were in Hardy Weinberg equilibrium in both groups of patients and healthy subjects.

Regarding the correlation between genotype and expression levels, significant results were obtained for SNPs −52G > A (p = 0.03 both in OLP patients and healthy individuals) and −44C > G (p = 0.02 in OLP patients (Table 1)).

Performing genetic models, we found that for −52G > A SNP in healthy subjects, the dominant model GG vs AG + AA was statistically significant (Kolmogorov-Smirnov test, p-value = 0.01) and for −44C > G SNP in OLP patients, model CC vs GC+ GG resulted statistically significant (Kolmogorov-Smirnov test, p-value = 0.01). Genetic models for other SNPs were not statistically significant.

Since in our previous work (Polesello et al., 2015) *DEFB1* 5′ UTR −52A and −44C SNPs have been reported as correlated with different hBD-1 salivary concentration, in this study the haplotypes analysis was also performed focusing on these two SNPs regardless of the others SNPs. *DEFB1* ACxxx haplotype (where x indicate any allele for −20G > A, c*5G > A and c*87A > G SNPs) were associated with low hBD-1 salivary concentration in healthy subjects (Table 2): individuals carrying the ACxxx/ACxxx combined haplotype presented lower hBD-1 salivary concentration compared to ACxxx/xxxxx and other subjects (Kruskal-Wallis test, p-value = 0.02, median 3.2 ng/ml, 5.18 ng/ml and 7.00 ng/ml, respectively; Table 2). Statistically significant results were obtained also comparing

ACxxx/ACxxx vs other and ACxxx/xxxxx vs other subjects but not for ACxxx/ACxxx vs ACxxx/xxxxx (p-value = 0.01, χ^2 = 2.06; df = 1; p-value = 0.047; p-value = 0.15, χ^2 = 2.06; df = 1 respectively (data not shown).

4. Discussion

hBD-1 is expressed by human salivary glands (Sahasrabudhe et al., 2000) and our study demonstrated that *DEFB1* SNPs at positions −52 and −44 could influence peptide production in saliva.

Recently, we observed on a limited number of individuals (n = 40) that *DEFB1* polymorphisms at position −52 and −44 were associated with the protein salivary concentration; −52 G/G

Table 2
Combined haplotypes for 5′UTR SNPs −52G > A, −44C > G, −20G > A and 3′UTR SNPs c*5G > A, c*87A > G in OLP patients and healthy individuals); only alleles −52A and −44C are considered ("x" means any other allele). Medians of hBD-1 concentrations for different haplotypes obtained with ELISA test and p-values are reported.

	OLP (n°=58) Median HBD-1 concentration (ng/ml)	OLP p-value (Kruskal Wallis test)	Healthy subjects (n° = 58) Median HBD-1 concentration (ng/ml)	Healthy subjects p-value (Kruskal Wallis test)
ACxxx/ACxxx	8.41 (n° = 10)	χ^2 = 0.89, df = 2, p-value = 0.64	3.2 (n° = 10)	χ^2 = 8.18, df = 2, p-value = 0.02*
ACxxx/xxxxx	11.66 (n° = 23)		5.18 (n° = 27)	
Other (i.e. xxxxx/xxxxx)	11.43 (n° = 25)		7.05 (n° = 21)	

*Statistically significant p-value.

carriers presented higher levels of hBD1 than G/A and A/A, while −44C/G subjects showed a higher protein concentration than homozygous wild-type C/C (only one subject was G/G and was not included in the analysis); combined haplotypes analysis confirmed the results obtained considering the SNPs singularly (Polesello et al., 2015). In this study we increased the number of healthy subjects to 58 and extended the analysis also to 58 OLP patients.

DEFB1 SNPs and hBD-1 concentration where analysed since it is known that in OLP patients oral keratinocytes are damaged (Dunsche et al., 2001; Mathews et al., 1999) and hBD-1 production could be compromised. In fact, significant result was obtained testing salivary concentrations of OLP versus healthy individuals (Kolmogorov-Smirnov test, $p = 0.00018$). To our knowledge, only one study was conducted on salivary hBD-1 and authors used Reversed Phase Liquid Chromatography to quantify hBD-1 in 30 patients with oral mucosal diseases, including 10 OLP, and compared concentration with 10 healthy volunteers (Kucukkolbasi, Kucukkolbasi, Ayyildiz, Dursun, & Kara, 2013); the authors showed higher significant hBD-1 production in patients than in healthy subjects, confirming our results.

Moreover we observed a statistically significant difference in hBD-1 production between −52 G > A genotypes in OLP patients and in healthy subjects (for both p-value = 0.03, Table 1): in agreement with our precedent observations among healthy subjects (Polesello et al., 2015), G allele seemed to influence protein production because subjects with G/G and G/A genotypes have a higher hBD-1 concentration. Dominant model GG vs AG + AA confirms the result (Kolmogorov Smirnov test, p-value = 0.01).

In OLP patients −44C/C genotype correlated with significantly lower production of hBD-1 than the other genotypes; for healthy subjects the result was not significant although the hBD-1 concentration was lower for C/C genotypes than C/G and G/G genotypes, in spite of our previous findings (Polesello et al., 2015). These results are in agreement with Kalus and co-workers' study (Kalus et al., 2009); they observed that the −44 G allele was correlated with increased constitutive and induced expression of *DEFB1* mRNA, using reporter constructs in oral keratinocytes cultured in vitro. In addition, the authors observed that constructs carrying the −44 G/G genotype had a more efficient antimicrobial activity than those with −44C/C (Kalus et al., 2009). These findings were in agreement with Sun's et al. study (Sun et al., 2006). Sun and co-workers showed that constructs with −44 G allele increased transcriptional activity using lucifease assay in DU145 and TSU-Pr1 cell lines (urogenital tumors cell lines) (Sun et al., 2006).

On the contrary different outcomes were obtained by Milanese et al., reporting a lesser hBD-1 production in cells that expressed −44 G plasmid, using Caco2 cell line and luciferase assay (Milanese et al., 2007; Sun et al., 2006). Considering the discordant and different results reported so far in the literature, further investigations are necessary to confirm the functional role of *DEFB1* regulatory SNPs on hBD1 production. Nevertheless it is important consider that the population studied in this work is not homogeneous in terms of age, because the average age of healthy subjects is lower than OLP patients (35 versus 65 years respectively) and age could influence hBD-1 production, since innate immunity is compromised with age (Solana et al., 2012). However the aim of our study was not a case-control study, but investigating the possible association between *DEFB1* polymorphisms and hBD-1 salivary levels, also considering OLP patients. The link between functional polymorphisms of *DEFB1* and the susceptibility to OLP, even if our findings seem to demonstrate a correlation, needs to be proven with other studies with major groups of subject with age matched controls.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors contribution

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 samples collection and manuscript revision; MG collected and prepared the samples; SC critically revised the manuscript; LS conceived the study and supervised the experiments and the analysis of the results. All authors have read and approved the final article.

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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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