DNMT3B (C46359T) Polymorphisms and Immunoexpression of DNMT3b and DNMT1 Proteins in Oral Lichen Planus

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Key Words

Oral lichen planus • Oral inflammatory fibrous hyperplasia • DNA methylation • DNMT3b polymorphism

Abstract

Objective: To investigate the *DNMT3B* (C46359T) polymorphism and immunoexpression of DNMT3b and DNMT1 in oral lichen planus (OLP) compared to a control group. **Methods:** We aimed to investigate the DNMT3B (C46359T) polymorphism and immunoexpression of DNMT3b and DNMT1 in OLP (n = 32), comparing it with oral mucosa (control; n = 24). The DNMT3B (C46359T) polymorphism was analyzed using the RFLP-PCR and DNMT1, and DNMT3a proteins were identified using immunohistochemistry. We also compared the DNMT3B expression in OLP and oral inflammatory fibrous hyperplasia (OIFH), another oral inflammatory disease. Differences between the groups were determined by specific statistical analyses. **Results:** The CT genotype of DNMT3B was associated with OLP development (p = 0.012). Increased expression of DNMT3B and DNMT1 was observed in OLP compared to the control group (p = 0.014 and p = 0.001, respectively). A significant increase in DNMT3B protein levels was observed in the genotype CT in *DNMT3B*

(C46359T) polymorphisms (p = 0.045). No DNMT3B expression differences between OLP and OIFH were observed. **Conclusions:** Our data show that the *DNMT3B* (C46359T) polymorphism is associated with OLP development. Furthermore, increased expression of the enzyme DNMT3B, an epigenetic-associated protein, is present in OLP.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory oral disease, the etiology of which is unknown but appears to be related to a cell-mediated immune process involving T lymphocyte aggression targeted to the basal layer of the oral mucosa [1–3]. The disease is more common in the female population and is most often seen in adults while rarely diagnosed in children [4, 5]. OLP may occur at any site of an oral cavity, but the oral mucosa, tongue and gingiva are more commonly affected sites. Clinically, the disease can manifest in several different forms: reticular (white lesions), erythematous (atrophic) and erosive (ulcerated, bullous) [3, 6, 7]. Evidence suggests that OLP is associated with an increased risk of de-

veloping cancer [8–10]. Although a number of studies have analyzed the malignant transformation of OLP, the specific process of malignization remains controversial [11–15]. Oral inflammatory fibrous hyperplasia (OIFH) is when reactive lesions appear, resulting from chronic oral irritation due to the use of poorly adapted dentures. Microscopically, OIFH consists of connective tissue characterized by collagen deposition and chronic inflammatory infiltration according to the stage of its development [16]. It was demonstrated that OIFH samples have similar characteristics to those observed in chronic inflammatory lesions [17].

Epigenetic modifications are phenomena that act non-specifically on DNA and can generate changes in the expression of any gene [18]. DNA methylation is the addition of methyl radicals to specific regions of DNA containing, predominantly, cytosine nucleotides. It is catalyzed by a family of enzymes denominated DNA methyltransferases (DNMTs), including three catalytically active enzymes – DNMT1, DNMT3a and DNMT3b [19–22]. DNMT3a and DNMT3b are responsible for de novo methylation and play an important role in controlling gene activity, embryonic development and genomic imprinting, while DNMT1 is an enzyme of maintenance and is thus involved in the establishment of a new methylation pattern [23, 24].

Although the functional effects of *DNMT3B* polymorphisms have not been elucidated, several studies have previously shown that they may affect DNMT3b protein activity during DNA methylation. It was reported that the single-nucleotide polymorphism SNP-149C→T in the promoter region of the *DNMT3B* gene was significantly associated with both higher promoter activity of the DNMT3b protein and a predisposition to aberrant methylation [25–27]. Given these previously published data, we attempted to analyze the *DNMT3B* (C46359T) polymorphisms and the expression of DNMT3b and DNMT1 proteins in OLP compared with the control. Moreover, we attempted to verify whether this *DNMT3B* (C46359T) polymorphism was associated with DNMT3b protein expression in OLP samples.

Patients and Methods

Study Design and Tissue Specimens

This cross-sectional study was performed on archived tissue blocks from surgically resected specimens of OLP (n = 32; male-to-female ratio = 1:1.9; mean age = 53.47 ± 3.60 years) and a control group (n = 24; male-to-female ratio 1:1.4; mean age = 32.83 years; SD 15.78 years; range 15–74 years). Mucocele samples were

used as a control group. We also compared DNMT3B expression with OIFH as a chronic inflammatory lesion (n = 8; male-to-female ratio = 1:0.6; mean age = 48.8 ± 9.8). Data related to patients and samples were obtained from the Department of Dentistry at the State University of Montes Claros, Minas Gerais, Brazil. All patients were from the same geographical area, but description of skin color was not used because, in Brazil, it is a poor predictor of genomic ancestry [28]. The diagnoses of patients presenting OLP lesions were confirmed by clinical examination and histopathological evaluation [29]. Ethical approval for this study was obtained from the relevant local ethics committees (Unimontes/Coep-1133/2008).

DNMT3B Genotypes

DNA was isolated from ten 10-μm-thick tissue sections from each tissue block of OLP specimens using the DNeasy Tissue Kit (Qiagen, Chatsworth, Calif., USA) as previously described [25].

DNMT3B (C46359T) polymorphism was assessed by RFLP with specific primers (forward 5'-TGGCTACCAGGTCTCCT-TGGCC-3'; 5'-GGTAGCCGGGAAGTCCACGG-3') [25]. Polymerase chain reaction for *DNMT3B* was performed in a total volume of 25 μl containing approximately 100 ng of genomic DNA template, 0.5 μl of each primer (20 pmol/μl), 2.5 μl dNTP mix (25 mM of each; AMRESCO, USA), 2.5 μl 10× PCR buffer, 1.25 μl magnesium chloride (50 mM), and 2.5 U of platinum Taq DNA polymerase (Invitrogen Life Technologies, USA). The 230-bp PCR product from the *DNMT3B* gene was digested with the *BlnI* restriction endonuclease (Sigma-Aldrich, USA), which recognizes a restriction site (C/CTAGG) in the T allele but not the wild-type C allele which lacks the *BlnI* restriction site. Thus, the wild-type C allele has only one band (230 bp), while the polymorphic T allele has two bands (172 and 58 bp). For this, 10 μl of amplified DNA was digested with 2.5 U of *BlnI* for 16 h at 37°C. PCR and restriction reactions were performed in a thermocycler (Eppendorf AG, Hamburg, Germany) and the PCR products were verified on a 6.5% polyacrylamide gel. DNA sequencing was performed to confirm the *DNMT3B* genotyping as determined by PCR-RFLP. All cases were classified according to the distribution of the genotypic polymorphisms for the *DNMT3B* gene (CC, CT, TT).

Immunohistochemical Analyses

For the immunohistochemical reactions, 3-μm-thick sections were mounted on organosilane-coated slides. Primary mouse monoclonal antibodies were used against DNMT3B (clone 52A1018, Imgenex, USA) and DNMT1 (clone 60B1220.1, 1:250, Imgenex). Antibodies were detected with the aid of an LSAB™ visualization kit (cat. No. K0690; Dako, Denmark) employing the chromogen diaminobenzidine for color development. Slides were finally counterstained with Mayer's hematoxylin and mounted. Negative controls were obtained by substituting the primary antibodies for normal whole rabbit serum (cat. No. X0902; Dako). A sample of oral carcinoma (previously shown to be strongly positive for the antibodies investigated) served as a positive control. Only cells that exhibited brown cytoplasmic or nuclear staining were considered positive.

Immunohistochemical Analysis

The immunohistochemical expression was evaluated using an Olympus® BH2 microscope (10× ocular and 40× objective lenses), and an ocular lattice (area 0.092 mm²) with 100 points com-

posed of 10 horizontal and 10 vertical test lines superimposed on the test field that was to be measured. A total area of 1.84 mm² was evaluated for each of the samples. Immunohistochemical analysis of DNMT1 and DNMT3b proteins was performed by obtaining a percentage of positive-staining cells in 20 microscopic fields (for each specimen).

Statistical Analysis

Results of protein expression were expressed as mean $\pm$ SD. Analysis of the (*C46359T*) polymorphisms in the control and the OLP were performed by χ^2 test with the Fisher and Pearson exact tests. The Kolmogorov-Smirnov test was used to determine whether the distribution of continuous variables was normal. Analysis of tissue immunolocalization of DNMT3b and DNMT1 assumed nonparametrical distribution and comparison between groups was done using the Mann-Whitney and Kruskal-Wallis statistical tests. All analyses were performed using SPSS 17.0 (SPSS Inc., USA) and statistical significance was set at $p < 0.05$. The concordance values obtained (OLP samples, $\kappa = 0.866$, $p < 0.05$) revealed good agreement.

Results

The impact of the *DNMT3B* polymorphism on the protein levels was evaluated with the Kruskal-Wallis test that showed a significant increase in DNMT3B protein levels associated with T allele (table 1).

The immunolocalization of DNMT3b and DNMT1 and the polymorphisms of the *DNMT3B* gene are shown in figure 1. Tissue expression of DNMT3b and DNMT1 were frequently noted in the basal and parabasal layers of all samples. However, this expression consistently demonstrated strong nuclear staining from the basal layer to the surface epithelium in the majority of the OLP cases. A higher tissue expression of DNMT3b ($p = 0.0140$) and DNMT1 ($p < 0.0010$) was noted in the OLP compared to the control (fig. 1; table 2). No differences in DNMT3B expression between LP and OIFH were observed (fig. 1). We also compared molecular features and clinical data (table 3), but no association between these factors was observed.

Discussion

Even with the increasing number of studies in the field of epigenetics, the clinical significance of these alterations in gene expression has not yet been established. DNA methylation is characterized by both the focal modification of gene-associated CpG islands and generalized genome-wide reduction in 5-methylcytosine, both of which are seen in various oral lesions, including cancer

Table 1. Association between the DNMT3B polymorphism and the expression of DNMT3B proteins in OLP and normal oral mucosa

Parameters	DNMT3B polymorphism		
	CC	CT	TT
Lesions			
OLP	6 (18.8%)	19 (59.4%)	7 (21.9%)
Oral mucosa	13 (54.2%)	10 (41.7%)	1 (4.2%)
p value		0.012	
Immunohistochemistry – mean			
DNMT3B	20.47	30.89	32.75
p value		0.045	
Significant p value < 0.05 is in bold. Analyzed by χ^2 and the Kruskal-Wallis test.			

Table 2. Association between different samples and expression of DNMT3B and DNMT1 proteins

	Lesions		
	OLP	oral mucosa	p value
Immunohistochemistry – mean			
DNMT3B	31.75	21.32	0.014
DNMT1	35.63	14.05	0.000
Significant p value < 0.05 is in bold. Analyzed by the Mann-Whitney test.			

and premalignant lesions [19, 25, 30]. This process can lead to a conformational change in the DNA and consequently generate modifications in the pattern of gene expression and silencing genetics [21, 31, 32].

Several studies suggest that the increased expression of DNA methyltransferases is associated with a predisposition to aberrant DNA methylation and, consequently, a change in gene expression [24, 33–35]. DNMT3b and DNMT1 proteins have been associated with the hypermethylation of several genes, many of which are related to processes associated with immunologically mediated diseases [36, 37].

In this study, we observed an increased expression of DNMT3b and DNMT1 in OLP when compared with control samples. Several studies have shown that DNA methylation regulates the expression of many different genes involved in the cell cycle and can, therefore, influence events pertaining to immunological disease [31, 36,

Fig. 1. Increased expression of DNMT1 protein (**a**) was found in OLP (**b**) compared to the control (**c**). * $p = 0.014$. **d** Higher expression of DNMT3b expression in OLP (**e**) and OIFH (**g**) compared to normal oral mucosa (OM) (**f**). # $p = 0.014$ and $^o p = 0.010$, respectively. No significant differences between OLP and OIFH were observed ($^+ p = 0.228$).

37]. Our data suggest that increased expression of these proteins in OLP may be related to DNA methylation events which consequently change the expression of several genes related to the immune response.

Single-nucleotide polymorphism (SNP) has been demonstrated in a vast majority of oral diseases [5, 38–41] and especially OLP [41–43]. It was postulated that the presence of the T allele increases DNMT3B protein expression and this leads to an increase in aberrant de novo methylation of CpG islands in some genes [35, 44]. Recently, various studies have shown that some SNPs in the *DNMT3B* gene may influence DNMT3b DNA methylation activity and might be associated with immunologically mediated diseases [44–47]. We showed that the CT genotype of the *DNMT3B* (C46359T) polymorphism was associated with OLP and, interestingly, this genotype was also associated with an increase in DNMT3B protein ex-

pression. A potential link among DNMT3B SNP, linked disequilibrium and the cytokine level might be involved in OLP or inflammation [41, 48]. In our previous study, we demonstrated that the T allele of DNMT3B genotype was significantly associated with advanced TNM staging and tumor size in older patients with head and neck cancer [25]. This fact may be associated with the malignant transformation of some cases of OLP.

Taken together, these data suggest that the *DNMT3B* (C46359T) polymorphism could alter the pattern of de novo DNA methylation due to increased expression of the DNMT3b protein and therefore influence the expression of genes related to OLP. Thus, DNA methylation in OLP seems to be a major event that may be related to the development of this lesion. Additionally, altered DNA methylation may also be associated with the malignant transformation of OLP, an idea supported by the

Table 3. Association between molecular features and clinical OLP

Parameters	Gender		Age		Clinical characteristics		Lesion site	
	male	female	≤45 years	≥46 years	reticular	atrophic/erosive	BM/RTM/T	P/FM
DNMT3B polymorphism								
CC	3 (50.0)	3 (50.0)	4 (66.7)	2 (33.3)	6 (100)	0	5 (83.3)	1 (16.7)
CT	6 (31.6)	13 (68.4)	7 (36.8)	12 (63.2)	15 (78.9)	4 (21.1)	16 (84.2)	3 (15.8)
TT	2 (28.6)	5 (71.4)	4 (57.1)	3 (42.9)	6 (85.7)	1 (14.3)	6 (85.7)	1 (24.3)
p value	0.664		0.366		0.462		0.993	
Immunohistochemistry – mean								
DNMT3B	17.14	16.17	14.33	18.41	16.15	18.40	16.74	15.20
p value	0.785		0.230		0.650		0.763	
<i>Mucocele analysis</i>								
DNMT3B polymorphism								
CC	3 (23.1)	10 (76.9)	12 (92.3)	1 (7.7)	–	–	–	–
CT	6 (60.0)	4 (40.0)	7 (70.0)	3 (30.0)	–	–	–	–
TT	1 (100.0)	0	0	1 (100.0)	–	–	–	–
p value	0.035		0.033		–		–	
Immunohistochemistry – mean								
DNMT3B	13.11	10.38	11.06	13.50	–	–	–	–
p value	0.357		0.538		–		–	
<i>Hyperplasia analysis</i>								
DNMT3B polymorphism								
CC	–	–	–	–	–	–	–	–
CT	–	–	–	–	–	–	–	–
TT	–	–	–	–	–	–	–	–
p value	–		–		–		–	
Immunohistochemistry – mean								
DNMT3B	5.33	4.00	4.00	4.80	–	–	–	–
p value	0.571		0.786		–		–	

Values in parentheses are percentages. Significant p value <0.05 is in bold. Analyzed by χ^2 and the Mann-Whitney test. BM = Buccal mucosa; RTM = retro-molar trigone mucosa; T = tongue; P = palate; FM = floor of mouth.

previously observed link between *p16^{CDKN2A}* methylation imbalance and oral carcinogenesis [19]. This assumption, however, needs to be demonstrated by further studies.

In conclusion, our data show that *DNMT3B* (C46359T) polymorphism is associated with the development of OLP. Furthermore, increased expression of the enzyme DNMT3B, an epigenetic-associated protein, is present in OLP.

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