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A 3′UTR polymorphism marks differential KLRG1 mRNA levels through disruption of a miR-584-5p binding site and associates with pemphigus foliaceus susceptibility

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ABSTRACT

Genetic variations mapping to 3′ untranslated regions (3′UTRs) may overlap with microRNA (miRNA) binding sites, therefore potentially interfering with translation inhibition or messenger RNA (mRNA) degradation. The aim of this study was to investigate whether single nucleotide polymorphisms (SNPs) located within the 3′UTRs of six candidate genes and predicted to interfere with miRNA ligation could account for disease-relevant differential mRNA levels. Focusing on pemphigus foliaceus (PF) – an autoimmune blistering skin condition with unique endemic patterns – we investigated whether nine 3′UTR SNPs from the *CD1D*, *CTLA4*, *KLRD1*, *KLRG1*, *NKG7*, and *TNFSF13B* genes differentially expressed in PF were disease-associated. The heterozygous genotype of the *KLRG1* rs1805672 polymorphism was associated with increased predisposition to PF (*A*/*G* vs. *A*/*A*: $P = 0.038$; OR = 1.60), and a trend for augmented susceptibility was observed for carriers of the *G* allele ($P = 0.094$; OR = 1.44). In silico analyses suggested that rs1805672 *G* allele could disrupt binding of miR-584-5p, and indicated rs1805672 as an expression Quantitative Trait Locus (eQTL), with an effect on *KLRG1* gene expression. Dual-luciferase assay showed that miR-584-5p mediated approximately 50% downregulation of the reporter gene's activity through the 3′UTR of *KLRG1* harboring rs1805672 *A* allele (vs. miRNA-negative condition, $P = 0.006$). This silencing relationship was lost after site-directed mutation to *G* allele (vs. miRNA-negative condition, $P = 0.391$; vs. rs1805672 *A* allele, $P = 0.005$). Collectively, these results suggest that a disease-associated SNP located within the 3′UTR of *KLRG1* directly interferes with miR-584-5p binding, allowing for KLRG1 mRNA differential accumulation, which in turn may contribute to pathogenesis of autoimmune diseases, such as pemphigus.

1. Introduction

Microribonucleic acids, or simply microRNAs (miRNAs), are short non-coding RNAs with a median size of 22 nucleotides (nt) in humans, once in their mature and posttranscriptional regulatory form [1,2]. The genes of these tiny molecules map to intergenic or intronic regions, where they are supposed to have their own promoter or share it with the host gene, respectively – i.e., intronic miRNAs are expected to be co-expressed with their host gene [3,4]. The mode by which miRNAs posttranscriptionally regulate gene expression through 3′ untranslated region (3′UTR)-binding of targeted messenger RNAs (mRNAs) is considered an RNA interference mechanism and leads to mRNA degradation or translation inhibition [1]. This sequence complementarity involves the so called miRNA seed sequence, a 6 or 7 nt 3′UTR-perfect matching region at the 5′-end of miRNAs that is known to be essential for effective miRNA function [1]. Interestingly, a new type of seed sequence located within the central region of miRNAs has been described [5], adding more complexity to the miRNA mode of action [6]. Regardless of where the seed sequence is located, it is clear that 3′UTR polymorphisms overlapping with seed sequences can interfere with miRNA ligation, potentially disrupting or creating miRNA binding sites [7].

Through gene expression profiles of complex traits, researchers can unveil new candidate genes for association studies and understand how genetic variation modulates normal and pathological conditions. Since our publication of the $CD4^+$ T cell genome-wide gene expression profile of pemphigus foliaceus [8], we have been interested in exploring how genetic polymorphisms influence gene transcription and mRNA stability and if these polymorphisms could be contributing to disease susceptibility [9].

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Pemphigus is a chronic autoimmune blistering skin disease identified by the presence of IgG autoantibodies (autoAb) directed against adhesion molecules of keratinocytes. Historically, the disease has been characterized by the presence of autoAb against two desmosomal cadherins, desmoglein (Dsg) 1 and/or Dsg3, believed to specify two main forms of the disease. Pemphigus foliaceus (PF) is identified by the presence of anti-Dsg1 autoAb and superficial blistering. The other main form, pemphigus vulgaris (PV), is recognized by the presence of anti-Dsg3 or anti-Dsg3 and anti-Dsg1 autoAb followed by suprabasal blistering of mucous membranes or by cutaneous and mucous involvement, respectively [10]. Additional self-antigens have been identified in pemphigus besides desmogleins: multiple other desmosomal and non-desmosomal cadherins, such as E-cadherin, and a large variety of human proteins that specifically react with pemphigus IgG [11,12]. The traditional understanding that anti-Dsg1 and/or anti-Dsg3 autoAb cause the loss of keratinocyte adhesion leading to blister formation (acantholysis) is being challenged and pemphigus is now more likely to be understood as a disease mediated by "apoptolysis" leading to keratinocyte shrinkage/detachment [13,14].

In epidemiologic terms, both main forms of pemphigus occur worldwide and sporadically. PF, however, has a form restricted to certain geographic regions, being the single known autoimmune disease with endemic features. Endemic pemphigus foliaceus (EPF) occurs in geographic areas of South America, such as in Brazil, where it was described in 1903 [15]. Brazilian pemphigus, also known as *fogo selvagem* ("wild fire", in literal translation), was estimated to reach 30.7 cases per million/year [16] and is believed to have clinical differences and prognostic dissimilarities to its non-endemic form, but no differences in immunopathology [15,17,18]. As the difference in endemicity may be due to environmental factors, we do not distinguish in this study individuals of EPF from those with the sporadic form.

Among the genes previously reported to be differentially expressed in PF, several are known for their central role in immune responses. For purposes of this study we selected six of these genes accounting for different types of immune responses (i.e., innate or adaptive, cellular or humoral). *CD1D* encodes the D member of the CD1 family of major histocompatibility complex (MHC)-like glycoproteins, responsible for capturing and presenting lipid and glycolipid antigens to T cells [19]. The cytotoxic T lymphocyte antigen-4 (*CTLA4*) gene is well known for encoding a negative regulator of T cell responses, contributing to the control of autoreactivity [20]. Killer cell lectin-like receptor subfamily D member 1 (*KLRD1* or *CD94*) and subfamily G member 1 (*KLRG1*) genes are both in the natural killer (NK) cell complex (NKC) in the chromosomal region 12p13. The former codes for a receptor establishing disulphide-bonded heterodimers with natural killer cell group 2 (NKG2) family members, acting together as activating or inhibitory NK cell receptors. The latter, *KLRG1*, codes for an inhibitory receptor expressed on the surface of mainly NK and $CD4^+$ and $CD8^+$ αβ T cells, where it potentially raises their activation thresholds, ultimately preventing autoreactivity [21–23]. The natural killer cell granule protein 7 (*NKG7*) gene is expressed in activated NK cells and possibly in some T lymphocyte subsets, where it may play a role in T-cell effector function [24]. Finally, the tumor necrosis factor superfamily member 13b (*TNFSF13B*) gene, commonly also referred as B cell activating factor (*BAFF*) or B lymphocyte stimulator (*BLYS*), codes for a cytokine important for the survival and proliferation of B and T cells [25].

In this context and considering pemphigus an interesting immune-related disease model, we investigated whether 3′UTR polymorphisms overlapping with predicted miRNA binding sites in the aforementioned PF-differentially expressed genes could be contributing to the previously observed mRNA variable levels. Through this approach, we describe an association between a *KLRG1* 3′UTR polymorphism and PF, and unveil a functional mechanism for the association that may also elucidate the KLRG1 mRNA differential levels previously observed in the disease.

2. Material and methods

2.1. Samples

Informed consent was obtained from all participants. This study was performed according to Brazilian federal laws and approved by the Human Research Ethics Committee of the Federal University of Paraná. Peripheral blood was collected for DNA extraction from 333 PF patients and 427 clinically healthy controls, most of these from the endemic area. Individuals were mostly contacted at Hospital Adventista do Pênfigo, in Campo Grande, Mato Grosso do Sul state. Some individuals were from: Curitiba (Hospital de Clínicas da UFPR, Hospital de Dermatologia Sanitária São Roque and Hospital Santa Casa de Misericórdia), in Paraná state; Ribeirão Preto (Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da USP), in São Paulo state; and Uberaba (Lar da Caridade - Hospital do Fogo Selvagem de Uberaba), in Minas Gerais state. As the Brazilian population is of mixed ethnic origin, to avoid effects of population structure, individuals were classified in two different groups for further sample adjustment: EU, of predominantly European origin; and M, of mixed European and African origin.

An additional sample of 146 healthy donors, contacted at the blood bank of the Hospital de Clínicas da UFPR, was included in this study for peripheral blood mononuclear cell (PBMC) isolation from venous blood by density gradient centrifugation with Histopaque® 1077 (Sigma-Aldrich Inc., Saint Louis, MO) according to manufacturer's manual.

2.2. Genotyping

Three different online-available bioinformatics tools were consulted to verify SNP ability to disrupt/create miRNA ligation: MirSNP, mirsnpscore, and PolymiRTS. Nine SNPs in the 3′UTR (Table 1) of six candidate genes previously shown to be differentially expressed in PF (Table S1) [8] were selected given their putative ability to disrupt/create a miRNA binding site (Table S2): rs116898958, rs185198828, and rs4145212 (*TNFSF13B*), rs3009 (*NKG7*), rs1805672 (*KLRG1*), rs2537752 (*KLRD1*), rs139105990 (*CTLA4*), rs16839951, and rs422236 (*CD1D*). All the aforementioned SNPs were predicted to fall within seed sequences and to disrupt/create a miRNA binding site according to at least one of the referred tools.

The *NKG7*, *KLRG1*, and *KLRD1* SNPs were genotyped by the SNPlex™ System (Applied Biosystems, Foster City, CA), which consists of an oligonucleotide ligation assay in a multiplex PCR, followed by capillary electrophoresis [26]. The *TNFSF13B* (*BAFF*), *CTLA4*, and *CD1D* SNPs were genotyped by iPLEX® MassARRAY® (Sequenom® Inc., San Diego, CA), which uses time of flight (TOF) mass spectrometry [27]. Further genotyping of the PF-associated rs1805672 SNP in PBMC of healthy donors was performed through TaqMan® SNP Genotyping Assay according to the manufacturer's protocol (Applied Biosystems).

Table 1
Genes and their 3′UTR SNPs analyzed in this association study.

Gene	Aliases	Location[a]	Analyzed SNPs	Reference variation[b]
CD1D	*CD1A*, *R3*	1q23.1	rs422236	*G* > *T*
			rs16839951	*A* > *G*
CTLA4	*ALPS5*, *CD152*	2q33.2	rs139105990	*G* > *A*
KLRD1	*CD94*	12p13.2	rs2537752	*T* > *A*
KLRG1	*2F1*, *CLEC15A*	12p13.31	rs1805672	*A* > *G*
NKG7	*GIG1*, *GMP-17*	19q13.41	rs3009	*G* > *A*
TNFSF13B	*BAFF*, *BLYS*	13q33.3	rs4145212	*T* > *A*
			rs116898958	*C* > *T*
			rs185198828	*G* > *T*

[a] Location is according to Ensembl genome browser.
[b] Forward to the genome according to dbSNP.