

RESEARCH NOTE

Polymorphism in ficolin-1 (*FCN1*) gene is associated with an earlier onset of type 1 diabetes mellitus in children and adolescents from northeast Brazil

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Introduction

The lectin pathway is activated by the mannose-binding lectin (MBL) and ficolins (FCN), both have already been associated with several autoimmune disorders. Here, we assessed *FCN1* and *FCN2* functional SNPs in 203 type 1 diabetes mellitus (T1D) patients with celiac disease (CD) and autoimmune thyroiditis (AITD). We identified that the *FCN1* rs1071583 SNP was correlated with earlier age of T1D diagnosis and the combination analysis from the SNPs (rs2989727 and rs1071583) assessed in *FCN1* and *FCN2* was associated with T1D lower susceptibility. This is the first finding that suggested the genetic role of *FCN1* and *FCN2* in northeastern Brazilian children and adolescents with T1D.

The lectin pathway of complement system acts in the elimination of pathogens, being able to phagocyte and induce inflammation response. The lectin pathway is activated by two different lectins, the mannose-binding lectin (MBL) and ficolins. So far, three humans ficolins are described: M-ficolin (ficolin-1), L-ficolin (ficolin-2) and H-ficolin (ficolin-3), encoded by the *FCN1*, *FCN2* and *FCN3* genes, respectively (Messias-Reason *et al.* 2009b; Hu *et al.* 2013). Several studies suggested the role of ficolins and MBL in the development of autoimmune disorders due to their ability to promote the apoptotic bodies' clearance, to increase the inflammation and to avoid viral infection (Atkinson *et al.* 2004; Vander Cruyssen *et al.* 2007; Messias-Reason *et al.* 2009a). In this study, we assessed the possible influence of *FCN1* and *FCN2* functional SNPs in T1D development and the insurgence of related AITD and CD.

Although *FCN1* and *FCN2* genes are located at the same chromosome region, 9q34 presents a differential expression pattern. *FCN1* is mainly expressed by leukocytes in intracellular secretory granules and is secreted in the interstitium and plasma. *FCN2* is expressed in liver cells and ficolin-2 protein once produced is secreted in the plasma (Garred *et al.* 2009).

Therefore, our hypothesis is that decrease in ficolins production/function in interstitium or plasma, related to the presence of such SNPs, located at regulatory region of *FCN1* and *FCN2* genes, may be responsible for apoptotic bodies' accumulation with a future autoimmune response in pancreatic tissues. Moreover, these SNPs in *FCN1* and *FCN2* could be involved with the autoantibody production in the AITD and CD in T1D patients.

Material and methods

Patients and healthy individuals

The study was carried out at three pediatric endocrinology reference services in Pernambuco, Brazil (Instituto de Medicina Integral Professor Fernando Figueira, Hospital da Restauração e Hospital das Clínicas), from March 2010 to December 2013. Children and adolescents were diagnosed with T1D according to American Diabetes Association criteria (2012). Ethics committee (Instituto de Medicina Integral Professor Fernando Figueira, number 1717/2010) approved the project and patients assigned a written free and informed consent.

We enrolled 204 T1D subjects (median age 13.5 years) diagnosed with T1D and 193 healthy individuals (median age 30 years), without clinical signs or family history of T1D and not related to the patient group, as controls. Both T1D

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patients and healthy individuals were born and reared in the same geographical region of Recife, Pernambuco, Brazil. As Brazilians are considered an admixed population, due to recent events of European and African migrations, we previously studied the ethnical composition of Recife population to avoid spurious association: we used 12 genetic ancestries in formative markers (AIMs) described by Kosoy *et al.* (2009), demonstrating a contribution of 59.7%, 23% and 17.3% of European, African and Native Amerindian ancestry, respectively (Coelho *et al.* 2015).

Diagnosis of autoimmune thyroid and celiac disease

Antibodies to thyroid peroxidase (anti-TPO) and antitransglutaminase (anti-tTG) were determined by chemio-luminescence (anti-TPO Ab, diagnostic products IMMULITE, Los Angeles) and by ELISA kit I-tTG (Eurospital, Trieste, Italy), respectively, following manufacturer's instructions.

DNA extraction

Genomic DNA was extracted from peripheral whole blood using Wizard Genomic DNA purification kit (Promega, Madison, USA) according to the standard laboratory protocols.

Polymorphism selection and genotyping

After *in silico* and literature search, we selected five SNPs: –1981 (rs2989727) and 7919 (rs1071583) located at promoter region and in exon 9 of *FCN1* gene, respectively, and –4 (rs17514136), +5839 (rs3124954) and +6424 (rs7851696) located at 5'UTR, intron 1 and exon 8 of *FCN2* gene, respectively. SNP in 5'UTR region of *FCN2* have been associated with variations in serum concentration of the protein, whereas the polymorphism located in exon 8, which encodes the domain similar to fibrinogen has been associated with an increase in the ability to link the acetylated residues of the protein (Luz *et al.* 2013). The rs3124954 represents a haplotype tagged by rs3128624 of *FCN2* gene.

The SNPs at promoter and exon 9 of *FCN1* could regulate both the expression and synthesis of ficolin-1 and were previously associated with autoimmune disorder (Vander Cruyssen *et al.* 2007). The rs1071583 of *FCN1* is synonymous SNP that changes dramatically the frequency of codon usage in the translation process, there by altering the rate of bioavailable protein and is associated with other autoimmune events (<http://www.kazusa.or.jp/codon/>).

Genotyping was performed using commercially available TaqMan probes (Applied Biosystem, Foster City, USA). The rs2989727 and rs1071583, by C_26745032_10 and C_1819018_1, respectively. The rs17514136, rs3124954 and rs7851696, by C_25765134_10, C_27462209_20 and C_29220549_20, respectively. TaqMan reactions were set up based on the manufacturer's protocol using 7500 Real-Time PCR instrument (Applied Biosystem).

Statistical analysis

Allelic discrimination was performed as suggested by the manufacturer (TaqMan[®] Genotyping Software), and

analysed using Genotyping Transposer software. Using the R statistical package (<https://www.r-project.org>), we obtained the chi-square test to correlate polymorphism distribution with the susceptibility to develop T1D and its clinical features as well as to evaluate the Hardy–Weinberg equilibrium (HWE). Linkage disequilibrium (LD) within *FCN1* and *FCN2* SNPs and haplotype constructions were assessed using the Haploview and SNPstats softwares (ver. 4.2). Odds ratio (OR) and 95% confidence intervals (CI) were also computed. All analyses, *P*-value <0.05 were considered statistically significant. Power analyses were performed using G*Power 3.1.9.2 software (<http://www.psych.uni-duesseldorf.de>). The association with the age of T1D onset was performed using the package SNPAssoc, implemented in statistical software R (ver. 3.0.0, <http://www.r-project.org>).

Results

In this study, a total of five SNPs, two SNPs of *FCN1* and three of *FCN2* genes were analysed in 204 T1D patients and 193 healthy subjects, classified according to the presence of other autoimmune disorder, namely CD and AITD.

Allele and genotype frequencies of *FCN1* gene polymorphisms (rs2989727 and rs1071583) were in HWE in both T1D patients and healthy individuals groups. The *FCN2* SNPs frequencies were also in HWE, except for rs7851696 (+6424 G>T variant) in both groups. No statistical differences were found in the SNPs distribution of T1D patients, CD and AITD subgroups and healthy individuals (*P* > 0.05) in all genetic models, i.e. codominant, dominant, recessive, overdominant and log-additive (data not shown). Only rs1071583 at *FCN1* was associated with age-at-diagnosis of T1D (*P*: 0.016; Akaike information criterion (AIC): 1095; dif: –2.20; 95% CI: –3.997 to –0.419; table 1). This association shows the genotype T/T as major susceptibility risk factor for an earlier insurgence of diabetes when compared to C/C and C/T genotypes. Patients with T/T genotypes could develop T1D around two years earlier than the others patients. No statistical association with the sex distribution was obtained for all five loci (*P* > 0.05, data not shown).

Since SNPs may act in combination to increase the risk of disease, the haplotypes of the studied *FCNs* SNPs were investigated and their frequencies in T1D and healthy control groups were compared. In spite of lack of strong LD between rs2989727 and rs1071583 SNPs (*D'* = 0.2), individuals carrying the C allele at both SNPs showed some protection against T1D onset (*P* value: 0.0003, OR: 0.53, 95% CI = 0.38–0.75) as shown in table 2.

Discussion

T1D is a multifactorial autoimmune disease caused by one or more environmental factors, such as viruses and bacterial toxins, interacting with the genetic profile of the individual

Table 1. Association between age of onset of type 1 diabetes and polymorphism in rs1071583 of *FCN1* gene.

	rs1071583	N	Av.	SD	Dif.	LL	UL	P value	AIC
Codominant	C/C	91	7.86	0.42	0.002	–	–	0.05	1097
	C/T	81	7.52	0.48	–0.34	–1.549	0.87		
	T/T	22	5.45	0.63	–2.37	–4.25	–0.49		
Dominant	C/C	91	7.86	0.42	0.00	–	–	0.19	1100
	C/T–T/T	103	7.09	0.41	–0.77	–1.92	0.37		
Recessive	C/C–C/T	172	7.70	0.32	0.00	–	–	0.02	1095
	T/T	22	5.49	0.63	–2.21	–3.98	–0.42		
Overdominant	C/C–T/T	113	7.40	0.37	0.00	–	–	0.84	1101
	C/T	81	7.52	0.48	0.12	–1.41	1.29		
Log additive	0.1.2	–	–	–	–0.91	–1.75	–0.06	0.04	1097

N, number of individuals; Av., average for a certain genotype; SD, standard deviation of average; Dif., the difference between the averages of certain genotype with genotype reference; LL, lower limit of the CI of difference; UL, upper limit of the CI of difference; AIC, Akaike information criterion.

Table 2. Frequencies of haplotypes in *FCN1* gene in T1D patients and healthy controls.

FCN1	rs2989727	rs1071583	HC	T1D	OR (95% CI)	P
1	T	C	0.2459	0.5050	1.00	–
2	C	T	0.1422	0.3216	0.94 (0.65–1.35)	0.72
3	C	C	0.4004	0.1734	0.53 (0.38–0.75)	3×10^{-4}
4	T	T	0.2116	0	0.00 (ND)	1

*Global haplotype association $P < 0.0001$; HC, healthy control; T1D, type 1 diabetes.

(Kyvik *et al.* 1995; Tsutsumi *et al.* 2003; Hansen *et al.* 2004). In the context of infectious triggering of T1D, it is known that ficolins can activate the lectin pathway of complement after binding to various microbial ligands such as mannose (Ohta *et al.* 1990), lipoteichoic acid (Lynch *et al.* 2004), GlcNAc (Matsushita *et al.* 1996), lipopolysaccharide (Neth *et al.* 2000; Zhao *et al.* 2002). Thus, it can be assumed that the reduction or deficiency of serum and interstitium ficolin-1/2 in children and adolescents could be a risk factors for T1D onset.

Further, as ficolins are involved in the cleaning of apoptotic bodies, its deficiency may also be associated with a poor removal of apoptotic cells resulting in the spread of autoantigens and immune system activation (Boniotto *et al.* 2005; Runza *et al.* 2008).

The knowledge of ficolin in autoimmune diseases remains scarce; in fact, in the literature, we found only one genetic association study with autoimmune disorder. Vander Cruyssen *et al.* (2007) described the association of rs2989727 and rs1071583 in *FCN1* with the development of rheumatoid arthritis. Both SNPs were included in the present study, but no association with T1D development was found. Further, these SNPs were not involved in the insurgence of CD or AITD or both. As Vander Cruyssen *et al.* (2007) demonstrated a strong LD between the SNPs rs2989727 and rs1071583, we also performed LD analysis and found that the combination of alleles C–C (for the rs2989727 and rs1071583, respectively) were associated with protection to T1D development, even if these SNPs were not in LD in our studied population. Unfortunately, Vander Cruyssen *et al.* (2007) did not perform the haplotype association, limiting our discussion.

The MBL and ficolin proteins may share the same molecular function and both take part in similar innate immune pathways as synonymous proteins, i.e., absence of one protein could be masked by the presence the other. Thus, we hypothesize that individuals carrying low levels of FCN1 or FCN2 could present normal or even high levels of MBL, thus creating a balance in the complement activation and apoptotic clearance.

Despite these major functions and similarities between MBL and ficolins, the ficolin-1 is the unique to be released by leukocytes that infiltrates the interstitium during the inflammation, such as is observed in the triggering T1D, which the beta-cells are infiltrated by reactive T cells generating an inflammatory process termed insulinitis. We believe that the absence of ficolin-1 in the microenvironment of the inflammatory process in pancreas tissues will not be balanced by the MBL presence. However, according to our results this hypotheses was not confirmed, corroborating Munthe-Fog *et al.* (2012) findings that demonstrated that the rs2989727 do not influence the *FCN1* expression in monocytes. Interestingly, children carrying the T/T genotype (rs1071583 of *FCN1*) presented an earlier age of diagnosis compared with genotypes C/C or C/T in a recessive model. The rs1071583 is a synonymous SNP at exon 9, that changes CAA to CAG, and the codon CAA has lower codon usage related to CAG (12.3 versus 34.2 per thousand of transcription) <http://www.kazusa.or.jp/codon/>, consequently, individuals carrying the genotype T/T (i.e. the codon CAA) could produce smaller quantity of ficolin 1. This finding suggests that *FCN1* is not directly implicated in triggering T1D onset but it could be involved in enhancing its consequences by creating a favourable environment for chronic conditions

in T1D patients. After the initial autoimmune response, the recruitment of leucocytes that produce a smaller quantity of ficolin 1 could diminish the apoptotic clearance and anticipating the autoimmune profile. However, further functional studies should be performed to clarify this hypothesis.

Conclusion

This is the first report to study the genetic role of *FCN1* and *FCN2* in northeastern Brazilian children and adolescents with T1D. *FCN1* rs1071583 SNP was correlated with earlier age of T1D diagnosis. In addition, the SNP combination rs2989727 and rs1071583 was involved with T1D protection. We are aware of the limitations of our study, related basically to the low number of individuals analysed as well as to the absence of functional validation (ELISA, Western, etc.) of the impact of *FCN1* and *FCN2* SNPs on the production and functionality of the proteins: thus both genetic replica and immunological functional studies should be done to increase the knowledge of *FCN* genes in the development of T1D.

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