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Meta-analysis of *STAT4* and *IFIH1* polymorphisms in type 1 diabetes mellitus patients with autoimmune polyglandular syndrome type III

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ABSTRACT. Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease characterized by T-cell mediated self-destruction of insulin-producing β cells in the pancreas. T1D patients are prone to develop other glandular autoimmune disorders, such as autoimmune thyroid disease that occurs simultaneously with autoimmune polyglandular syndrome type III (APSIII). Signal transducer and activator of transcription 4 (STAT4) is a well-known regulator of proinflammatory cytokines, and interferon-induced with helicase C domain 1 (IFIH1) is activated in the interferon type I response. Both genes have been examined separately in autoimmune diseases and,

in this study, we assessed their joint role in T1D and APSIII. We conducted a case-control study, enrolling 173 T1D patients and 191 healthy controls from northeastern Brazil, to assess the distribution of the rs7574865 and rs3024839 SNPs in *STAT4* and the rs3747517 and rs1990760 SNPs in *IFIH1* in T1D and APSIII patients. Additionally, we conducted a meta-analysis with the rs7574865 SNP in *STAT4* (1392 T1D patients and 1629 controls) and the rs1990760 SNP in *IFIH1* (25092 T1D patients and 28544 controls) to examine their association with T1D. Distribution of *STAT4* and *IFIH1* allelic frequencies did not show statistically significant differences between T1D patients and controls in our study population; however, the meta-analysis indicated that SNPs in *STAT4* and *IFIH1* are associated with T1D worldwide. Our findings indicate that although *STAT4* and *IFIH1* SNPs are not associated with T1D in a Brazilian population, they might play a role in susceptibility to T1D on a larger worldwide scale.

Key words: STAT4; IFIH1; Autoimmune polyglandular syndrome type III; SNP; Type 1 diabetes

INTRODUCTION

Type 1 diabetes mellitus (T1D) is an organ-specific autoimmune disease characterized by T cell-mediated attack of the insulin-producing β cells in the pancreas, leading to insulin deficiency (Gillespie, 2006). T1D is a multifactorial disease caused by genetic and environmental factors, as well as their interaction, that play a key role in the development of the disease. In up to one-quarter of T1D patients, an unbalanced immune system leads to autoimmune polyglandular syndrome type III (APSIII), which is characterized by the simultaneous occurrence of autoimmune thyroid disease (AITD) and sex bias (adult females are preferentially affected) (Kordonouri et al., 2002; Dittmar and Kahaly, 2010). Furthermore, patients with APSIII may also be diagnosed with celiac disease (CD). Despite the genetic variation in human leukocyte antigen (HLA) and its involvement in T1D development, new genes have been identified as potentially important in disease's susceptibility and modulation outside HLA range (Gillespie, 2006; Liang et al., 2012).

Signal transducer and activator of transcription 4 (STAT4) is a latent cytoplasmic transcription factor activated by phosphorylation in response to proinflammatory cytokines, such as interleukin (IL)-12, IL-15, and IL-23 (Levy and Darnell, 2002). STAT4 is involved in T helper 1 (Th1) cell regulation and is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at inflammation sites. Additionally, STAT4 mediates IL-12 signaling, which modulates Th1 cell differentiation and proliferation, interferon-γ (INF-γ) production, and development of T helper 17 (Th17) cells (Kobayashi et al., 2008; Zervou et al., 2008). Since Th1 cells are critical effectors of chronic inflammation disorders, STAT4 could play a pivotal role in the pathogenesis of immune diseases (Kobayashi et al., 2008; Zervou et al., 2008; Bi et al., 2013; Zheng et al., 2013). In fact, single nucleotide polymorphisms (SNPs) within *STAT4* (chromosome location: 2q32.2-q32.3) have been reported to be associated with increased risk for several autoimmune diseases (Liang et al., 2012), including rheumatoid arthritis (RA) (Stark et al., 2009), systemic lupus erythematosus (SLE) (Kobayashi et al., 2008), and Sjögren's syndrome (SS) (Palomino-Morales et al., 2010).

Viral infections have been implicated as triggers in autoimmune disorders in T1D (Jun and Yoon, 2001; Salminen et al., 2003). Interferon-induced with helicase C domain 1 (IFIH1; gene located at chromosome 2q24), also known as MDA5, activates the type I interferon (IFN-I) pathway and pro-inflammatory cytokines by its CARD domains after detecting double-stranded RNA viruses (Chistiakov, 2010). Enterovirus infections, particularly coxsackievirus B4 strains, are known to be T1D-associated; therefore, IFIH1 may play an important role in the development of T1D and its autoimmune-related disorders (Jaïdane et al., 2009). Interestingly, during IFN-I activation, IFIH1 and STAT4 share a common pathway and, since both genes are known to be associated with T1D (Zheng et al., 2013), one can hypothesize that defects in both genes may increase susceptibility to disease compared to defects in just one gene, resulting in a cumulative effect of genetic mutations.

In this study, we investigated the single nucleotide polymorphisms (SNPs) rs7574865 (G > T) and rs3024839 (T > C) in *STAT4* and rs3747517 (C > T) and rs1990760 (C > T) in *IFIH1* and their link to T1D and APSIII susceptibility in a northeast Brazilian population. Additionally, we performed a meta-analysis for the rs7574865 and rs1990760 SNPs in T1D predisposition.

MATERIAL AND METHODS

Patients and control subjects

We performed a case-control study in T1D patients from Pernambuco State in northeast Brazil. We enrolled 173 T1D patients ranging in age from 0 to 18 years at diagnosis, with an age of 7.3 ± 4.1 (means ± SD) years at disease onset. The patients attended one of three pediatric endocrinology departments in the public healthcare system in Recife, Brazil (Instituto de Medicina Integral Professor Fernando Figueira, Hospital da Restauração and Hospital das Clínicas). A consent form was obtained from all patients (or their legal representative) enrolled in this study. T1D patients were diagnosed according to American Diabetes Association (ADA) criteria and classified as T1D from clinical and pathological presentation (Gabir et al., 2000).

From the T1D patient group, 47 (27.2%) were diagnosed with APSIII. AITD was diagnosed using antibodies against thyroperoxidase (anti-TPO) and detection was performed using chemiluminescence (Immulite anti-TPO, Diagnostic Products Co., Los Angeles, CA, USA) following the manufacturer instructions. The individuals positive for TPO (titer exceeding 35 IU/mL, according to indications provided by the manufacturer) were considered as presenting AITD. The control group consisted of 191 healthy unrelated blood donors from the same geographic region with no history of autoimmune or chronic disease. The age of the control group ranged from 16 to 72 years and the means ± SD age was 38.8 ± 14.7 years. This study was carried out with advanced approval from the local Ethics Committee (IMIP Nos. 762/2006 and 1717/2010).

Genotyping

Genomic DNA was obtained from whole blood and the extraction protocol was performed according to the manufacturer instructions (Wizard Genomic DNA Purification Kit; Promega, Madison, MA, USA). The DNA was stored at -20°C until analysis. The SNPs assessed in this study

have frequently been described in the literature: rs7574865 (G > T) from intron 3, rs3024839 (T > C) from exon 4 in *STAT4*, rs3747517 (C > T) in exon 13, and rs1990760 (C > T) in exon 15 of *IFIH1*. Genotyping was performed using commercially available Taqman probes and the ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The allelic discrimination protocol was performed as recommended by the manufacturer and analyzed using SDS 2.3 software (Applied Biosystems).

Statistics and meta-analysis

Association analyses were performed by the chi-squared (χ^2) test with continuity correction, and the odds ratio (OR) and 95% confidence intervals (CI) were calculated using the Fisher test. For the meta-analysis, we searched peer-reviewed articles published between 2003 and 2015 (last search performed in July 2015) using logical equations with the following key words on PubMed and Web of Knowledge: "[(IFIHI or MDA5) and T1D]" or "(STAT4 and T1D)". We selected only case-control studies with the allele counts available for both SNPs analyzed (rs7574865 and rs1990760). The meta-analysis tests were carried out using the "Metafor" package (Viechtbauer, 2010). When the P value from the Cochran Q test for heterogeneity was lower than 0.1, the DerSimonian-Laird's estimator was used for the random-effect or fixed-effect models when necessary. The Haploview version 4.2 software was used for calculation of haplotype associations. Power analyses were performed using the G*Power 3.1.3 software (http://www.psycho.uniduesseldorf.de).

RESULTS

The allele and genotype frequencies of the SNPs in *STAT4* and *IFIH1* in patients (T1D + AITD + CD, T1D only, and APSIII) and healthy controls are shown in Table 1. All polymorphisms were in Hardy-Weinberg equilibrium in all groups except the T1D only group. Distribution of *STAT4* and *IFIH1* genotype and allele frequencies did not show statistically significant differences between patients and controls, indicating no association of the SNPs with development of T1D or APSIII regardless of the genetic model used. These results are shown in Table 2. Of note, the examined SNPs did not show linkage disequilibrium.

Furthermore, we performed a meta-analysis for the rs7574865 and rs1990760 SNPs from *STAT4* and *IFIH1*, respectively. Including the present study, we gathered seven publications with data on the rs7574865 SNP (Lee et al., 2008; Martínez et al., 2008a; Zervou et al., 2008; Howson et al., 2011; Park et al., 2011; Bi et al., 2013) and ten for the rs1990760 SNP (Smyth et al., 2006, 2008; Martínez et al., 2008b; Aminkeng et al., 2009; Jermendy et al., 2009; Liu et al., 2009; Schulte et al., 2010; Yang et al., 2012; Bouças et al., 2013; Zurawek et al., 2015). The total number of cases and controls was 1392 and 1629 for the rs7574865 SNP and 25,092 and 28,544 for the rs1990760 SNP, respectively.

The forest plots of the meta-analyses of the rs7574865 and rs1990760 SNPs are shown in Figures 1 and 2, respectively. The rs7574865 (OR = 1.37; 95%CI = 1.23-1.52; P < 0.0001) and rs1990760 (OR = 0.85; 95%CI = 0.81-0.89; P < 0.0001) SNPs were both associated with T1D, although some moderate heterogeneity was detected (I^2 = 43.35%; $P_{|Q|}$ = 0.0478) for the rs1990760 SNP.

Table 1. Allele and genotype frequencies of the *STAT4* (rs7574865 and rs3024839) and *IFIH1* (rs3747517 and rs1990760) SNPs in type 1 diabetes mellitus patients (T1D only), T1D patients with autoimmune thyroid disease (AITD) and celiac disease (CD), autoimmune polyglandular syndrome type III (APSIII) patients, and healthy controls (HC).

SNP	HC		T1D + AITD + CD		T1D only		APSIII	
	N	Freq.	N	Freq.	N	Freq.	N	Freq.
rs7574865								
G	294	0.77	260	0.75	197	0.78	63	0.68
T	88	0.23	86	0.25	57	0.22	29	0.32
GG	112	0.59	98	0.57	76	0.60	22	0.48
GT	70	0.37	64	0.37	45	0.35	19	0.41
TT	9	0.05	11	0.06	6	0.05	5	0.11
rs3024839								
T	353	1.00	331	1.00	245	1.00	86	1.00
C	1	0.00	1	0.00	1	0.00	0	0.00
TT	176	0.99	165	0.99	122	0.99	43	1.00
TC	1	0.01	1	0.01	1	0.01	0	0.00
CC	0	0.00	0	0.00	0	0.00	0	0.00
rs3747517								
C	230	0.68	249	0.72	179	0.72	70	0.74
T	110	0.32	95	0.28	71	0.28	24	0.26
CC	77	0.45	87	0.51	63	0.50	24	0.51
CT	76	0.45	75	0.44	53	0.42	22	0.47
TT	17	0.10	10	0.06	9	0.07	1	0.02
rs1990760								
C	202	0.61	201	0.60	150	0.61	51	0.57
T	130	0.39	135	0.40	96	0.39	39	0.43
CC	59	0.36	67	0.40	52	0.42	15	0.33
CT	84	0.51	67	0.40	46	0.37	21	0.47
TT	23	0.14	34	0.20	25	0.20	9	0.20

Table 2. Odds ratios, 95% confidence intervals, and P values from the association analysis between the *STAT4* (rs7574865 and rs3024839) and *IFIH1* (rs3024839 and rs1990760) variants and type 1 diabetes (T1D) and autoimmune polyglandular syndrome type III (APSIII).

Comparison	11 x 01 x 00			01 x 00			11 x 00			1 x 0
	P value	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
rs7574865										
HC *vs* T1D	0.7737	1.04	(0.68-1.61)	0.9302	1.40	(0.56-3.51)	0.6320	1.11	(0.79-1.55)	0.6258
HC *vs* T1D only	0.9753	0.95	(0.59-1.52)	0.9184	0.98	(0.34-2.87)	0.8095	0.97	(0.66-1.41)	0.9371
HC *vs* APSIII	0.1864	1.38	(0.7-2.73)	0.4507	2.83	(0.86-9.25)	0.1570	1.54	(0.93-2.54)	0.1188
T1D only *vs* APSIII	0.2038	1.46	(0.71-2.98)	0.3947	2.88	(0.8-10.34)	0.1910	1.59	(0.94-2.7)	0.1127
rs3024839										
HC *vs* T1D	ND	1.07	(0.09-23.29)	0.5067	ND	ND	ND	1.07	(0.07-17.12)	0.5073
HC *vs* T1D only	ND	1.44	(0.09-23.29)	0.6444	ND	ND	ND	1.44	(0.09-23.15)	0.6449
HC *vs* APSIII	ND	0.00	ND	0.4415	ND	ND	ND	0.00	ND	0.4420
T1D only *vs* APSIII	ND	0.00	ND	0.5812	ND	ND	ND	0.00	ND	0.5817
rs3747517										
HC *vs* T1D	0.2983	0.87	(0.53-1.38)	0.6264	0.52	(0.22-1.2)	0.1821	0.80	(0.57-1.11)	0.2046
HC *vs* T1D only	0.5700	0.85	(0.53-1.38)	0.6000	0.65	(0.27-1.55)	0.4438	0.83	(0.58-1.18)	0.3480
HC *vs* APSIII	0.2176	0.93	(0.48-1.8)	0.9589	0.19	(0.02-1.49)	0.1519	0.72	(0.43-1.2)	0.2539
T1D only *vs* APSIII	0.4346	1.09	(0.55-2.16)	0.9433	0.29	(0.04-2.43)	0.4108	0.86	(0.5-1.48)	0.6929
rs1990760										
HC *vs* T1D	0.1037	0.70	(0.37-1.04)	0.1805	1.30	(0.69-2.45)	0.5124	1.04	(0.77-1.42)	0.8486
HC *vs* T1D only	0.0688	0.62	(0.37-1.04)	0.0941	1.23	(0.63-2.43)	0.6645	0.99	(0.71-1.39)	0.9569
HC *vs* APSIII	0.5943	0.98	(0.47-2.06)	0.8848	1.54	(0.59-4.01)	0.5259	1.19	(0.74-1.9)	0.5512
T1D only *vs* APSIII	0.5036	1.58	(0.73-3.43)	0.3298	1.25	(0.48-3.24)	0.8351	1.19	(0.73-1.95)	0.5567

0 represents the less frequent allele and 1 is the most frequent; ND = not determined; OR = odds ratio; CI = confidence interval; HC = healthy control.

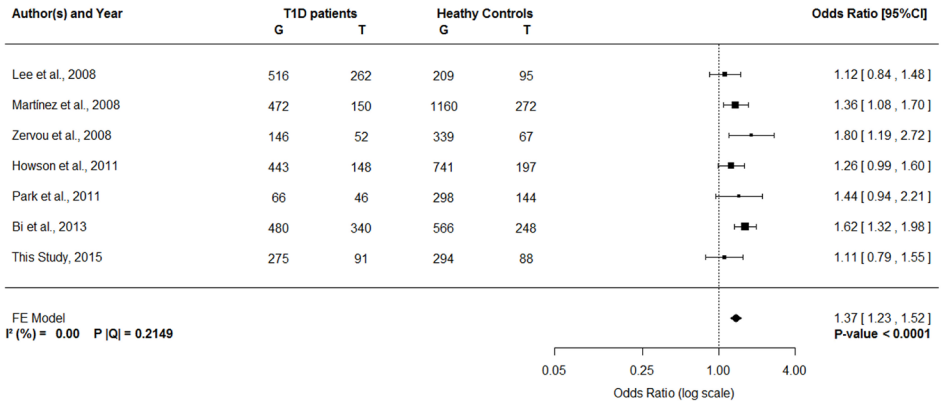

Figure 1. Forest plot from the meta-analysis for the SNP rs7574865 within the *STAT4*.

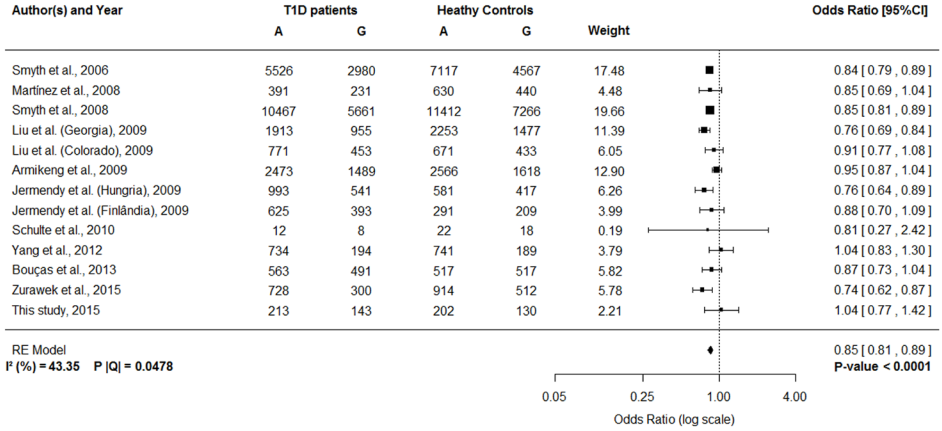

Figure 2. Forest plot from the meta-analysis for the SNP rs7574865 within the *IFIH1*.

DISCUSSION

Several T1D patients present simultaneously with autoimmune disorders and organ commitment. APSIII is the most common autoimmune disorder in T1D patients and is found more frequently in adults; it has low incidence in children. The individual pathogenic mechanisms underlying T1D, AITD, and CD remain unknown. Furthermore, it is unclear whether the development of these diseases is due to a shared common etiopathological mechanism or if it is just a consequence of the presence of one autoimmune disorder functioning as a trigger for the insurgence of another. The regulatory pathway of T cells might be a molecular target to understand the pathogenesis of APSIII due to the involvement of T cell activation in T1D, AITD and CD mechanisms. In addition, the role of viral infection and its related pathways may also be important in understanding the link between T1D, AITD, APSIII, and CD.

In this study, we assessed the role of *STAT4* and *IFIH1* variants in T1D and APSIII susceptibility. *STAT4* is involved in Th1 regulation and its inhibition prevents the development of

autoimmune diabetes in non-obese diabetic (NOD) mice. Moreover, genetic variants of *STAT4* are associated with autoimmune disorders in several populations, making STAT4 an emerging therapeutic target (Yang et al., 2004; Bi et al., 2013; Zheng et al., 2013). *IFIH1* SNPs were first associated with T1D in autoimmune disease and have since been associated with other autoimmune systemic disorders. Upon viral infection, IFIH1 activates the IFN-I pathway and the release of IFN-I can activate STAT4, followed by the Th1 gene expression profile in mature dendritic cells (Kariuki et al., 2008). Herein, we investigated the individual and combined influence of *STAT4* and *IFIH1* variants on T1D and APSIII development. In addition, we examined if *STAT4* and *IFIH1* SNPs are associated with development of T1D in a meta-analysis study.

The rs7574865 SNP is one of the most frequently examined polymorphisms in the *STAT4* gene and its function is related to gene expression on a transcriptional level and splice variation (Liang et al., 2012). The T allele of this particular SNP has been associated with multiple autoimmune disorders but the association is dependent on the population examined (Lee et al., 2010). In the present study, the SNPs examined from *STAT4* and *IFIH1* did not show any correlation (individually or combined) with incidence of T1D and/or APSIII. Although the rs7574865 SNP is within intron 3, it displays a linkage disequilibrium with other SNPs that have a possible functional consequence (Zheng et al., 2013). The rs3024839 SNP is an intragenic missense mutation (A > G resulting in isoleucine > valine substitution) with probable functional consequences in STAT4. Although *STAT4* SNPs have been frequently studied as potential indicators for autoimmune diseases, the results are still unclear, indicating that STAT4 might play varying roles in these disorders. Our results agree with the meta-analysis performed by Zheng et al. (2013), which revealed that the *STAT4* rs7574865 polymorphism is associated with several autoimmune diseases, including SLE, RA, scleroderma, systemic sclerosis (SSc), and primary SS, but is not associated with T1D, ulcerative colitis, and Crohn's disease. Interestingly, SLE, RA, SSc, and SS are considered systemic disorders, whereas T1D is characterized as an organ-specific manifestation, which suggests that mutations in *STAT4* are primarily related to systemic rather than organ-specific disorders.

On the other hand, the study performed by Zervou et al. (2008) assessed the link between the rs7574865 SNP and risk of T1D in Crete, where there is a genetically homogenous population, and the results indicated that there was an association. Moreover, this polymorphism was strongly associated with T1D in a northeastern Chinese population (Bi et al., 2013). Additionally, the study performed by Fourati et al. (2012) assessed the possible role of non-HLA genes in APSII, which includes Addison's disease and AITD and/or T1D, in a Tunisian population. Their results indicated that the rs7574865 SNP in *STAT4* was associated with APSII but not with T1D or AITD alone, suggesting that *STAT4* is involved with the co-occurrence of autoimmune endocrinopathies in APSII individuals.

IFIH1 is a helicase that senses viral dsRNA and, when activated, supports the transcription of IFN-I and IFN-induced genes (Robinson et al., 2011). Since IFIH1 acts during viral infections, we hypothesized in a previous publication that a defective mechanism in virus recognition might be caused by a defective IFIH1 (Moura et al., 2013). However, in the present study, we did not find any association between the rs3747517 (A > G resulting in histidine > arginine substitution in exon 13) or rs1990760 (C > T resulting in alanine > threonine substitution in exon 15) SNPs in IFIH1 and T1D or APSIII. Despite this, on including our data in the meta-analysis study, the rs1990760 SNP in *IFIH1* was found to be associated with T1D. The overexpression of IFIH1 in murine models is related to a chronic state of IFN-I production (Crampton et al., 2012). In multiple sclerosis (MS), which is an autoimmune disease, *IFIH1* and Toll-like receptor 7 (*TLR7*) are overexpressed. TLR7

is associated with the IFN-I response and, consequently, the IFN signature (Hundeshagen et al., 2012). The T allele in the rs1990760 SNP in *IFIH1* is associated with increased expression of IFIH1 in peripheral blood mononuclear cells and sensitivity to IFN-α (Rönnblom et al., 2011). Therefore, both STAT4 and IFIH1 exert some control in the IFN-I pathway, and their malfunction leads to an altered immune response.

In conclusion, we did not find any association of SNPs in *STAT4* or *IFIH1* with T1D development in a northeast Brazilian population. However, the meta-analysis showed an association between the rs7574865 and rs1990760 SNPs in *STAT4* and *IFIH1*, respectively, with T1D, even when our negative associations were included.

Conflicts of interest

The authors declare no conflict of interest.

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