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ORIGINAL ARTICLE

TIM-3 polymorphisms in type 1 diabetes families

Patrick Brück · Elizabeth Ramos-Lopez · Wiebke Bartsch · Angelika Böhme · Klaus Badenhoop

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Abstract TIM-3 is a transmembrane protein preferentially expressed on differentiated Th1 cells, which play a role in Th1-mediated diseases including type 1 diabetes. We investigated the role of the rs10515746 (A/C), rs1036199 (A/C), and rs10053538 (A/C) single nucleotide polymorphisms (SNPs) within the *TIM-3* gene in 186 German type 1 diabetes families (558 individuals) and its interaction with human leukocyte antigen (HLA) high-risk haplotypes *DQ2(DQA*0501-DQB*0201)-DQ8 (DQA*0301-DQB*0302)*. Alleles A, C, and A of the rs10515746 (A/C), rs1036199 (A/C), and rs10053538 (A/C) SNPs were found in a frequency of 20.4%, 19.0%, and 4.2%, respectively. Transmission analysis of these polymorphisms did not show any significant difference. Although in patients with HLA *DQx/x* (neither HLA *DQ2* nor *DQ8*) an undertransmission of allele A (14.3% vs. 85.7%) of the rs10053538 (A/C) SNP and an overtransmission of allele A (66.7% vs. 33.3%) of the rs10515746 (A/C) SNP was observed, these associations did not remain statistically significant after correction for multiple comparisons. Although we found no association of TIM-3 with type 1 diabetes in the German population, we cannot discard a possible association in a larger size.

P. Brück · A. Böhme
Department of Internal Medicine II,
Division of Haematology and Oncology,
University Hospital Frankfurt,
Frankfurt am Main, Germany

E. Ramos-Lopez · W. Bartsch · K. Badenhoop (✉)
Department of Internal Medicine I, Division of Endocrinology,
Diabetes and Metabolism, University Hospital Frankfurt,
Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
e-mail: badenhoop@em.uni-frankfurt.de

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Introduction

Organ-specific T-lymphocyte-mediated inflammation may lead to type 1 diabetes and other autoimmune diseases (Roep 2003), where a breakdown in immunological self-tolerance and a Th1-cell-mediated response against self-antigens is observed. The balance of Th1 and Th2 cells is critical in the pathogenesis of diverse autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes, in which a predominance of Th1 can induce autoimmunity, whereas a Th2 predominance can be protective (Romagnani 1994; Kamradt et al. 2001; Kuchroo et al. 1995).

The differentiation of naive T cells into Th1 or Th2, as well as the extent of their activation, is determined by the duration and strength of T-cell-receptor (TCR)-mediated stimulation and by the influence of tumor necrosis factor (TNF) receptor, immunoglobulin (Ig) superfamily members, and cytokines (Kündig et al. 1996; Locksley et al. 2001; Salomon et al. 2001; Refaeli et al. 1998).

Previous reports indicate that the family of T-cell immunoglobulin domain and mucin domain (TIM) proteins are expressed on T cells (Umetsu et al. 2005). In humans, the *TIM* gene family, located on chromosome 5q33.2, consists of *TIM-1*, *TIM-2,* and *TIM-3* genes, which encode cell-surface glycoproteins with common structural motifs, including signal peptides, Ig domains, mucin domains, transmembrane regions, and intracellular tails with phosphorylation sites (Kuchroo et al. 2003). TIM-3 is a transmembrane protein preferentially expressed on differentiated Th1 cells, which appears to play an essential role

in Th1-mediated immune responses and in macrophage activation (Monney et al. 2002). In nonobese diabetic (NOD) mouse with spontaneous insulin-dependent diabetes, treatment with TIM-3-specific monoclonal antibodies accelerated the onset of disease (Sanchez-Fueyo et al. 2003). In cerebrospinal-fluid-derived mononuclear cells of patients with multiple sclerosis, TIM-3 messenger RNA (mRNA) expression correlated with high expression of interferon gamma (IFN-γ) (Khademi et al. 2004). Furthermore, polymorphisms in the coding and promoter region of the *TIM-3* gene were identified and associated with rheumatoid arthritis in a Korean population (Chae et al. 2004a, b).

Therefore, we investigated the role of the following TIM-3 polymorphisms in German type 1 diabetes families: the rs10053538 (A/C) and rs10515746 (A/C) single nucleotide polymorphisms (SNPs) in the promoter region of the *TIM-3* gene at positions –1516 and –574, respectively, as well as the rs1036199 (A/C) SNP mapped to exon 3, resulting in an amino acid substitution (Arg → Leu) (Chae et al. 2004a, b). There are another 105 additional SNPs described in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) that were not studied (Fig. 1).

Furthermore, we analyzed the distribution of TIM-3 polymorphisms according to the presence or absence of the human leukocyte antigen (HLA) risk haplotypes [DQ2 (DQA1*0501-DQB1*0201) and DQ8 (DQA1*0301-DQB1*0302)] to detect a possible interaction with HLA or an independent association in the small risk-negative subgroup.

Subjects and methods

In the family analysis, where the transmission of the four parental haplotypes can be followed unambiguously and the nonaffected parental haplotypes originate from the same population as the disease sample, the sampling problem is eliminated, and thereby, both the disease sample and an appropriate control are obtained. Another advantage of a family analysis is parallel evaluation of the inheritance and allows indirect proof of the accuracy of the genotype methods (Falk et al. 1987). For these reasons, ours was a family-based analysis.

Subjects

Type 1 diabetes families ($n = 186$) comprising both parents and a single affected offspring ($n = 558$ subjects) were enrolled in this study. All families were recruited from the endocrine outpatient clinics at the University Hospital Frankfurt am Main, Germany. Type 1 diabetes was diagnosed according to World Health Organization (WHO) criteria. The female:male ratio of affected siblings was 1:1.3, and the median age at the diagnosis was 11.5 years. All individuals were of German origin. The study protocol was approved by the Ethics Committee of the University Hospital Frankfurt am Main, Germany, and written informed consent was obtained of all individuals.

Genotyping

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit® (Quiagen, Hilden, Germany) according to manufacturer's instructions. The rs10515746 (A/C) and rs1036199 (A/C) TIM-3 polymorphisms were analyzed by real-time polymerase chain reaction (PCR) using the ABI 7300 (Applied Biosystems, Darmstadt, Germany). Primer and probe sequences were as follows for the rs10515746 (A/C) SNP: forward primer 5′- ACAGT GAATGGCATGTTCCTTATCC-3′, reverse primer 5′-GA AGGATGAGAGTGAGGCTTATGC-3′; probe for A allele 5′-VIC- ATTTACAGACCATAGCAACT-3′; probe for C allele 5′-FAM- TTACAGACCATCGCAACT -3′. For the rs1036199 (A/C) SNP: forward primer 5′- CTTGGAAA GGCTGCAGTGAAG-3′; reverse primer 5′- TGTTGTTTC TGACATTAGCCAAGGT-3′; probe for A allele 5′-VIC-TCTCTCTGCAGAGTCG-3′; probe for C allele 5′-FAM-TCTCTCTGCCGAGTCG-3′.

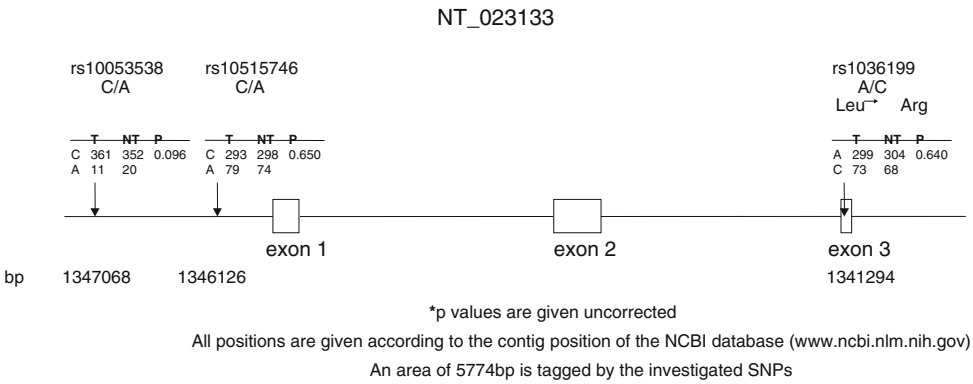

Fig. 1 The studied TIM-3 polymorphisms and their relative position in the gene

The rs10053538 (A/C) was studied by restriction fragment length polymorphism (RFLP) using the forward primer 5′-ACCACCCCGGATAATTTTGT-3′and reverse primer 5′-GCCTTGACCAAGTTCATGCT-3′ for the PCR. The resulting 404-bp fragment was digested with *BsI I*, generating two fragments of 330 and 74 bp after digestion in the presence of the C allele. Fragments were separated on 2% agarose gel and visualized by ethidium bromide. To prove the accuracy of the applied methods (standard and real-time PCR), 100 DNA samples—chosen at random—were genotyped for each polymorphism. The methods demonstrated an accuracy of 99.7%.

SNP selection

So far, no data correlating TIM-3 polymorphisms and type 1 diabetes have been reported. We concentrated our analysis on polymorphisms, which were previously associated with the autoimmune disease rheumatoid arthritis (Chae et al. 2004a, b).

HLA genotyping

Since HLA *DQ2* (*DQA1*0501-DQB1*0201*) and HLA *DQ8* (*DQA1*0301-DQB1*0302*) are known to be the strongest type 1 diabetes associated HLA haplotypes (Donner et al. 1997, 2000), families ($n = 160$) were grouped according to the offsprings' HLA *DQ* genotype into two group: those with the haplotype *DQ2/DQ8* heterozygous, *DQ2* and *DQ8* homozygous, and genotype *DQ2*/x or *DQ8*/x—considered as a one group—and those without HLA high-risk genotypes *DQx/x* (neither *DQ2* nor *DQ8*).

Statistical analysis

SNP and haplotype frequencies as well as the linkage disequilibrium (LD) between markers were estimated using Haploview software version 3.2 available from http://www.broad.mit.edu/mpg/haploview, whereas the correlation with HLA risk alleles [*DQ2* (*DQA*0501-DQB*0201*)–*DQ8* (*DQA*0301-DQB*0302*)] was evaluated using Unphased software version 2.403 available from http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/

Power calculation was performed assuming an allele frequency of 4.2% (TIM-3 38 A), 19.0% (TIM-3 99 C), and 20.6% (TIM-3 46 A), respectively (derived from parents used in this study) and a type 1 error rate of 5%. On the basis of these assumptions, we estimate that our study has a statistical power of only 11.8%, 31.2%, and 32.7%, respectively, to detect an allelic odds ratio (OR) of 1.5 for disease susceptibility in the family data set. All power estimates were done with PBAT (version 3.5) (Lange et al. 2004). Estimates were based on approximation and were calculated for an additive disease model. *P* values were corrected by the number of comparisons ($n = 26$). A corrected *P* (P_c) < 0.05 was considered significant.

Results

No deviations from Hardy–Weinberg equilibrium were observed in parents or affected sibling in each polymorphism. Rs10515746 and rs1036199 SNPs were in strong LD with each other ($r^2 = 0.887$), whereas rs10053538 was not in LD with rs10515746 ($r^2 = 0.0040$) or with rs1036199 ($r^2 = 0.0030$).

Allele A of the rs10053538 (A/C) SNP was found with a frequency of 4.2%, allele A of the rs10515746 (A/C) SNP in 20.6%, and allele C of rs1036199 (A/C) in 19.0%, respectively. Analysis of the transmission and nontransmission of these SNPs showed no significant difference (Table 1). No difference in the transmission of these polymorphisms was found in relation to patient gender (data not shown).

Analysis of haplotypes rs10515746A-rs10053538A and rs1036199C-rs10053538A revealed that both were not transmitted (0 vs. 3 times, $P = 0.0411$, Table 2) from parents to their affected offspring. The same pattern was observed for the haplotype rs1036199C-rs10515746A-rs10053538A (0 vs. 3 times, $P = 0.0411$, $P_c > 0.05$, Table 2). However, after correction for the multiple testing, these differences did not remain statistically significant.

Table 1 Transmission (T) and nontransmission (NT) of TIM-3 polymorphisms in 186 type 1 diabetes families

Polymorphism	Allele	Frequency (%)	T (%)	NT (%)	*P** value	OR (95% CI)
rs10053538	C	95.8	361 (50.6)	352 (49.4)	0.0964	1.86 (0.88–3.95)
	A	4.2	11 (35.5)	20 (64.5)		0.54 (0.25–1.14)
rs10515746	C	79.4	293 (49.6)	298 (50.4)	0.6501	0.92 (0.65–1.31)
	A	20.4	79 (51.6)	74 (48.4)		1.09 (0.76–1.55)
rs1036199	A	81.0	299 (49.6)	304 (50.4)	0.6400	0.92 (0.63–1.32)
	C	19.0	73 (51.8)	68 (48.2)		1.09 (0.76–1.58)

OR odds ratio, *CI* confidence interval

**P* values are given uncorrected

Table 2 Haplotype analysis of TIM-3 polymorphisms in 186 type 1 diabetes families

Haplotype	Frequency (%)	T(%)	NT (%)	*P**	OR (95% CI)
rs10053538- rs10515746					
CC	75.7	282 (50.1)	281 (49.9)	0.9319	1.01 (0.73–1.42)
AC	20.2	79 (52.7)	71 (47.3)	0.4647	1.14 (0.80–1.64)
CA	3.7	11 (39.3)	17 (60.7)	0.2460	0.64 (0.29–1.38)
AA	0.4	0	3 (100)	0.0411[a]	–
LOD = 0.71; $D' = 0.609$ (0.1 – 0.86); $r^2 = 0.0040$					
rs1036199 - rs10053538					
AC	77.3	288 (50.1)	287 (49.9)	0.9303	1.02 (0.72–1.43)
CC	18.5	73 (52.9)	65 (47.1)	0.4504	1.15 (0.80–1.67)
AA	3.8	11 (39.3)	17 (60.7)	0.2460	0.64 (0.29–1.38)
CA	0.4	0	3 (100)	0.0411[a]	–
LOD = 0.54; $D' = 0.568$ (0.08–0.84); $r^2 = 0.0030$					
rs1036199 - rs10515746					
AC	79.3	293 (49.7)	297 (50.3)	0.7174	0.94 (0.66–1.34)
CA	18.9	73 (52.1)	67 (47.9)	0.5735	1.11 (0.77–1.61)
AA	1.7	6 (46.2)	7 (53.8)	0.7795	0.85 (0.28–2.57)
CC	0.1	–	1 (100)	–	–
LOD = 128.94; $D' = 0.991$ (0.95–1.0); $r^2 = 0.887$					
rs1036199- rs10515746-rs10053538[b]					
ACC	75.5	282 (50.2)	280 (49.8)	0.8646	1.03 (0.74–1.44)
CAC	18.5	73 (53.3)	64 (46.7)	0.3945	1.17 (0.81–1.70)
ACA	3.8	11 (39.3)	17 (61.0)	0.2460	0.64 (0.29–1.38)
AAC	1.7	6 (46.2)	7 (53.8)	0.7795	0.85 (0.28–2.57)
CAA	0.4	0	3 (100)	0.0411[a]	–
CCC	0.1	0	1 (100)	–	–

T transmission, *NT* nontransmission, *OR* odds ratio, *CI* confidence interval, *LOD* logarithm of odds

**P* values are given uncorrected

[a] Not significant after correction

[b] Haplotypes that were not present (AAA and CCA) were not listed

Furthermore, no differences were found for haplotype rs10515746-rs1036199 (Table 2).

Of the studied families, 28 (17.5%) offspring carried haplotype *DQx/x* (neither *DQ2* nor *DQ8*), and the remainder of families (82.5%) were found to have HLA high-risk genotypes. Interaction analysis between TIM-3 polymorphisms and HLA haplotypes revealed that patients with haplotype *DQx/x* had an undertransmission of allele A (14.3% vs. 85.7%, $P = 0.0410$) of the rs10053538 (A/C) polymorphism but an overtransmission of allele A (66.7% vs. 33.3%) of the rs10515746 (A/C) polymorphism. Thus, rs10053538 allele A significantly protected in the cohort without HLA high risk (*DQx/x)* and tended to be protective in the whole cohort. Nevertheless, no association was observed after correction ($P_c > 0.05$) for multiple testing. No association was observed for the rs1036199 polymorphism in HLA risk-negative families (Table 3). Due to the low number of patients in the different subgroups, we did not perform a haplotype analysis.

In patients with HLA high-risk genotypes [*DQ2 (DQA*0501-DQB*0201)–DQ8 (DQA*0301-DQB*0302)*], distribution of the three TIM-3 polymorphisms did not differ significantly (Table 3).

Discussion

This is the first report investigating the role of TIM-3 polymorphisms in type 1 diabetes. TIM-3 is a potential candidate gene to type 1 diabetes susceptibility for several reasons: TIM3 is a molecule expressed on Th1 cells, where it is critical for macrophage activation (Umetsu et al. 2005). Furthermore, the treatment of NOD mice with TIM-3 monoclonal antibodies accelerates the onset of diabetes (Sanchez-Fueyo et al. 2003), and TIM-3 is located on chromosome 5q33, a region that has been implicated as a susceptibility locus. Regulatory interleukin (IL)-12B alleles, also mapped to 5q33-34, were found to

Table 3 Transmission (T) and nontransmission (NT) of TIM-3 polymorphisms from parents to offspring according to human leukocyte antigen (HLA) haplotype

Polymorphism	Allele	Non-HLA high-risk *DQx/x*[a]					HLA high-risk DQ2/DQ8 [b]				
		Frequency (%)	T (%)	NT (%)	P	OR (95% CI)	Frequency (%)	T (%)	NT (%)	*p*	OR (95% CI)
rs10053538	C	94.4	61 (52.1)	56 (47.9)	0.0410	6.54 (0.76–55.9)	94.7	287 (50.2)	285 (49.8)	0.7240	1.14 (0.56–2.33)
	A	5.6	1 (14.3)	6 (85.7)		0.15 (0.02–1.31)	5.3	15 (46.9)	17 (53.1)		0.88 (0.43–1.79)
rs10515746	C	73.2	36 (43.9)	46 (56.1)	0.0316	0.39 (0.16–0.94)	79.9	224 (50.8)	217 (49.2)	0.4572	1.17 (0.77–1.78)
	A	26.8	20 (66.7)	10 (33.3)		2.56 (1.06–6.13)	20.1	52 (46.8)	59 (53.2)		0.85 (0.56–1.30)
rs1036199	A	78.6	40 (45.5)	48 (54.5)	0.0634	0.42 (0.16–1.07)	80.7	222 (50.6)	217 (49.4)	0.5869	1.13 (0.73–1.72)
	C	21.4	16 (66.7)	8 (33.3)		2.40 (0.93–6.19)	19.3	50 (47.6)	55 (52.4)		0.89 (0.58–1.36)

OR odds ratio, *CI* confidence interval

[a] *DQx/x*: (neither *DQ2* nor *DQ8*)

[b] *DQ2/DQ8*: *DQ2/DQ8* heterozygous, *DQ2* and *DQ8* homozygous and genotype *DQ2*/x or *DQ8*/x

be preferentially transmitted to the affected offspring (Morahan et al. 2000). This susceptibility locus was named IDDM18, and IL-12B alleles were differently expressed in Epstein-Barr virus (EBV) cell lines. However, a study of Danish, European, and American families could not confirm any association, although the correlation of IL-12 alleles and expression showed a trend of a higher IL-12 secretion in the presence of the 1159C allele (Bergholdt et al. 2004). Still, a genome scan finds little support of the IDDM18 locus on chromosome 5q33, with a maximum logarithm of odds (LOD) score of just below 1 (Concannon et al. 2005), which may be due to the fact that susceptibility is either weak or operational in some populations only.

In this study, we investigated the role of three polymorphisms within the TIM-3 gene in German type 1 diabetes families. In accordance with a previous study, the frequencies observed in type 1 diabetes were similar to those observed in patients with rheumatoid arthritis (Chae et al. 2004a, b). Nevertheless, we could not find a direct association between polymorphisms within the TIM-3 gene and type 1 diabetes.

Although it was not significant after correction for multiple comparisons, there was a protective trend for allele A of the rs10053538 in the whole cohort and a significant protective effect in *DQx/x* patients. Allele frequencies of these polymorphisms varied in the studied population between 4.2% and 20.4%.

HLA haplotypes represent the strongest genetic risk markers to type 1 diabetes in diverse populations. In a large study in German and Belgian type 1 diabetes families, 13.4% of families were HLA DQx/x (Pani et al. 2002). In our study population, only 17.5% of type 1 diabetes families carried no HLA high-risk genotype (HLA DQx/x). Given that the HLA risk genotype is found in the majority of the patients with type 1 diabetes, our analysis was forcibly based on a small group, and the links between TIM-3 polymorphisms, HLA, and type 1 diabetes need to be elucidated in further studies of larger size.

Still, the possible association of TIM-3 polymorphisms in type 1 diabetes with *DQx/x* (non-*DQ2/DQ8* heterozygous, non-*DQ2*, and non-*DQ8* homozygous or genotype *DQ2*/x or *DQ8*/x) may point to susceptibility in this subset of patients (She 1996). Nevertheless, as our HLA analysis has little statistical power, the possible association of TIM-3 polymorphisms in HLA DQx/x allows a limited interpretation of these findings.

As demonstrated by the administration of TIM-3 immunoglobulin fusion proteins during a Th1-cell-mediated immune response leading to hyperproliferation of Th1 cells and increased production of Th1-type-cytokines (Sabatos et al. 2003), TIM-3 normally inhibits Th1-cell effector responses.

A dysfunction of TIM-3 could explain why patients develop type 1 diabetes in absence of an HLA-risk allele. As the rs10053538 (A/C) and rs10515746 (A/C) polymorphisms are located in the promoter region, whereas the rs1036199 (A/C; Arg → Leu) is on exon 3 of the TIM-3 gene, the observed polymorphic allele change could result in a different regulation of TIM-3 function, with a subsequently disturbed differentiation of Th1/Th2 cells and/or dysregulation of macrophage activation.

Additionally, the role of TIM-3 in a range of inflammatory conditions—such as insulitis—has recently been reported, where an antibody agonist of TIM-3 acted as an adjuvant during immune response and TIM-3 ligation induced distinct signaling events in T-cells (Anderson et al. 2007).

Although there are limitations of this study due to the restricted number of families with low statistical power and only three SNPs genotyped, TIM-3 is an excellent candidate gene for type 1 diabetes, not only because of its potential role in inflammatory conditions, but also because TIM-3 SNPs were associated with another autoimmune disease.

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