The Review of DIABETIC STUDIES

ORIGINAL DATA

Posttranslational Protein Modifications in Type 1 Diabetes - Genetic Studies with *PCMT1*, the Repair Enzyme Protein Isoaspartate Methyltransferase (PIMT) Encoding Gene

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■ Abstract

BACKGROUND: Posttranslational protein modifications have been implicated in the development of autoimmunity. Protein L-isoaspartate (D-aspartate) O-methyltransferase (PIMT) repairs modified proteins and is encoded by *PCMT1*, located in a region linked to type 1 diabetes (T1D), namely *IDDM5*. AIM: To evaluate the association between genetic variations in the *PCMT1* gene and T1D. METHODS: Firstly, *PCMT1* was sequenced in 26 patients with T1D (linked to *IDDM5*) and 10 control subjects. The variations found in *PCMT1* were then tested (alone and interacting with a functional polymorphism in *SUMO4* and with *HLA*) for association with T1D in 253 families (using transmission disequilibrium test). In a third step, the association of the functional variation in *PCMT1* (rs4816) with T1D was analyzed in 778 T1D patients and 749 controls (using chi-square test). *In vitro* promoter activity was assessed by transfecting INS-1E cells with *PCMT1* promoter constructs and a reporter gene, with or without cytokine stimulation. RESULTS: Four polymorphisms in complete linkage disequilibrium were identified in *PCMT1* (5' to the gene (rs11155676), exon 5 (rs4816) and exon 8 (rs7818 and rs4552)). In the whole cohort of 253 families, the allele associated with increased PIMT enzyme activity (rs4816, allele A) was less frequently transmitted to the affected than to the non-affected offspring (46% vs. 53%, p = 0.099). This finding was even more evident in the subset of families where the proband had high-risk *SUMO4* (p = 0.069) or low-risk *HLA* (p = 0.086). Surprisingly, in the case-control study with 778 cases and 749 controls, an inverse trend was found (40.36% of patients and 36.98% of controls had the allele, p = 0.055). *PCMT1* promoter activity increased with cytokine stimulation, but no differences were detected between the constructs adjacent to rs11155676. CONCLUSION: *PCMT1* was virtually associated with T1D in groups defined by other risk genes (*SUMO4* and *HLA*). A general association in a not further defined sample of T1D patients was not evident. Verification in a larger population is needed.

Keywords: type 1 diabetes · INS-1E cells · promoter activity · protein isomerisation · polymorphism · SUMO4

Introduction

Posttranslational protein modifications may create new antigenic epitopes and elicit an autoimmune response. In fact, many of these modifications have been shown to play a role in both animal models and human autoimmune disease [1-5]. We proposed that cytokines, produced by macrophages and dendritic cells in the islet of Langerhans, might cause posttranslational protein modifications triggering multiple antigen-specific B- and T-lymphocyte responses in the initiation phase of the pathogenesis of

type 1 diabetes (T1D) [6]. In addition, we recently published data supporting a role for the repair enzyme protein L-isoaspartate (D-aspartate) O-methyltransferase (PIMT) in the pathogenesis of T1D. It is highly expressed in human beta-cells, and an inducer of this enzyme delays diabetes onset and reduces the severity of the disease in diabetes-prone BB rats [7]. *Pcmt* knock-out mice display an autoimmune phenotype, with increased T-cell proliferative responses to mitogen and receptor-mediated stimulation. Furthermore, anti-DNA auto-antibodies develop in wild-type mice transferred with bone marrow from animals lacking *Pcmt* [8].

In humans, the gene encoding PIMT (*PCMT1*) is located on chromosome 6q24-25, in a genomic region which has been previously linked to T1D (*IDDM5*) [9].

The aim of this study was to further elucidate the role of PIMT in T1D through assessing the results from *PCMT1* genetic studies. These included screening the gene for variations in a selected group of individuals, assessing their potential functional implications and evaluating the association between these genetic variations in the *PCMT1* gene (on their own or through their interaction with other genes) and T1D.

Methods

Subjects

For mutational scanning of the *PCMT1* gene, a group of 26 patients with T1D, in whom the disease was linked to the region of interest (*IDDM5*), and 10 non-affected subjects were selected from a cohort of 147 Danish multiplex families with T1D [10]. In order to genotype the variants found in this initial screening, DNA from 253 families with type 1 diabetes (1097 individuals) was used; 155 sib-pair families and 98 simplex families, including the 147 families mentioned above [11]. The same individuals were genotyped for a functional variant in *SUMO4* (rs237025) and for *HLA*. The former is located less than 1 cM from *PCMT1*, and has been proposed to be associated with T1D [12, 13], whereas *HLA* is the major genetic risk determinant for T1D [14].

All probands were less than 30 years of age at the time of diagnosis. All participants gave written informed consent for the performance of the study, which had been approved by the appropriate local Ethics Committee.

Association of *PCMT1* with T1D was also assessed in 778 T1D patients and 749 controls obtained from a large Danish population-based case-control study [15].

PCMT1 sequencing and genotyping

For the family study, DNA was extracted from peripheral blood leukocytes using a standard salting-out method. *PCMT1* sequencing primers were based on the reference sequence available from the National Center for Biotechnology Information (NCBI; Bethesda, MD, http://www.ncbi.nlm.nih.gov) and the University of California Santa Cruz Genome (http://www.genome.ucsc.edu) databases. Primers were designed to give PCR products covering the gene's 8 exons (1800 base pairs) and 800 base pairs upstream of exon 1. Each PCR product (210-550 nucleotides) was amplified and sequenced on automated sequencing equipment (ABI 3100, Applied Biosystems, Foster City, CA, USA) using Big Dye terminator 3 chemistry (Applied Biosystems). Data were analyzed using SeqScape version 2.0, Applied Biosystems.

The variants identified in *PCMT1* during initial sequencing were subsequently genotyped in the cohort of 253 Danish families, by PCR-based restriction fragment length polymorphism, using the enzymes Pvu II, BseM II, Hga I and Afl II for the polymorphisms in the 5' region, exon 5 and exon 8, respectively.

In the case-control study, genotyping of rs4816 was performed on a Sequenome platform (RSKC2 core facility) on whole genome amplified DNA [16]. The following primers were used: first primer: ACG TTG GAT GTG GAT CGT CCT TCC TGA CAT, second primer: ACG TTG GAT GGT ACT GGA AAA GTC ATA GG, mass extension primer: CAC CGT AAA GAG CTA GTA GAT GAC TCA. PCR programmes followed instructions provided by Sequenom (Sequenom, San Diego, CA, USA).

SUMO4 and HLA genotyping

The functional variant in the *SUMO4* gene (rs237025, 55Met/Val) previously shown to be associated with T1D [12] was genotyped using Tetra-primer ARMS-PCR [17]. Briefly, two primer pairs were used to amplify the two alleles of a given SNP in a single PCR reaction, yielding three different PCR products: a non-specific product, determined by the "outer primers" and two allele-specific products, determined by each specific "inner" primer and its "outer" counterpart. HLA-DRB1 genotype was determined by allele-specific PCR for the DR3 and DR4 alleles [10].

Genetic analysis

A combined Transmission Disequilibrium Test (TDT) sib-TDT was performed [18] to determine the

association of the variants found in *PCMT1* and T1D, as well as for the functional variant in *SUMO4*. Furthermore, data on *PCMT1* was stratified according to the proband's *SUMO4* genotype, as well as risk *HLA* genotype. DR3/DR4 was defined as high-risk *HLA* [14], whereas all other genotypes were classified as non-high risk *HLA*. Parent homozygocity TDT [19] was also performed to assess the interaction between *SUMO4* and *PCMT1*. For this purpose, parents with *SUMO4* genotype 55Val/Val, previously defined as high-risk for the development of T1D [12], were selected. In addition, the number of high-risk alleles was compared in affected and non-affected individuals.

In the case-control study, association of rs4816 to T1D was tested with allelic and genotypic tests, using a chi-squared test with 1 and 2 degrees of freedom, respectively.

Functional studies

Information available from both the National Center for Biotechnology Information (NCBI; Bethesda, MD, http://www.ncbi.nlm.nih.gov) and the University of California Santa Cruz Genome (http://www.genome.ucsc.edu) genomic databases and previously published data were reviewed to assess the potential functional consequences of the variations found in *PCMT1*.

In silico analysis of the 5' region in search of potential binding sites for transcription factors was performed using several open access, web-based programmes: Alibaba 2.1 (www.alibaba2.com), Genomatix MatInspector (www.genomatix.de/cgi-bin/ matinspector) and Genomatix PromoterInspector (www.genomatix.de/cgi-bin/promoterinspector).

In vitro promoter activity was assessed using 8 promoter constructs cloned upstream of the firefly luciferase (*luc*) reporter gene, comprising 1817-201 nucleotides upstream of the starting codon of *PCMT1* in exon 1 (Figure 1). All constructs were verified by sequencing and restriction enzyme cleavage. INS-1e cells (passage numbers 65-89) were co-transfected with each reporter construct and renilla-luciferase as an internal control (pRL-TK vector, Promega, Madison, WI, USA, catalogue #E2241). Transfections were performed using a cationic lysosome-based method (SuperFect, Qiagen, Ballerup, Denmark), in the absence or presence of IL-1 (150pg/ml), IFNγ (5ng/ml) or both. Luciferase activity was measured using an Orion micro-plate luminometer (Berthold Technologies, Bad Wildbad, Germany). The ratio of firefly/renilla luciferase activity was calculated for each construct and treatment. Five experiments were performed, with each condition in duplicate. Each treatment and pro-

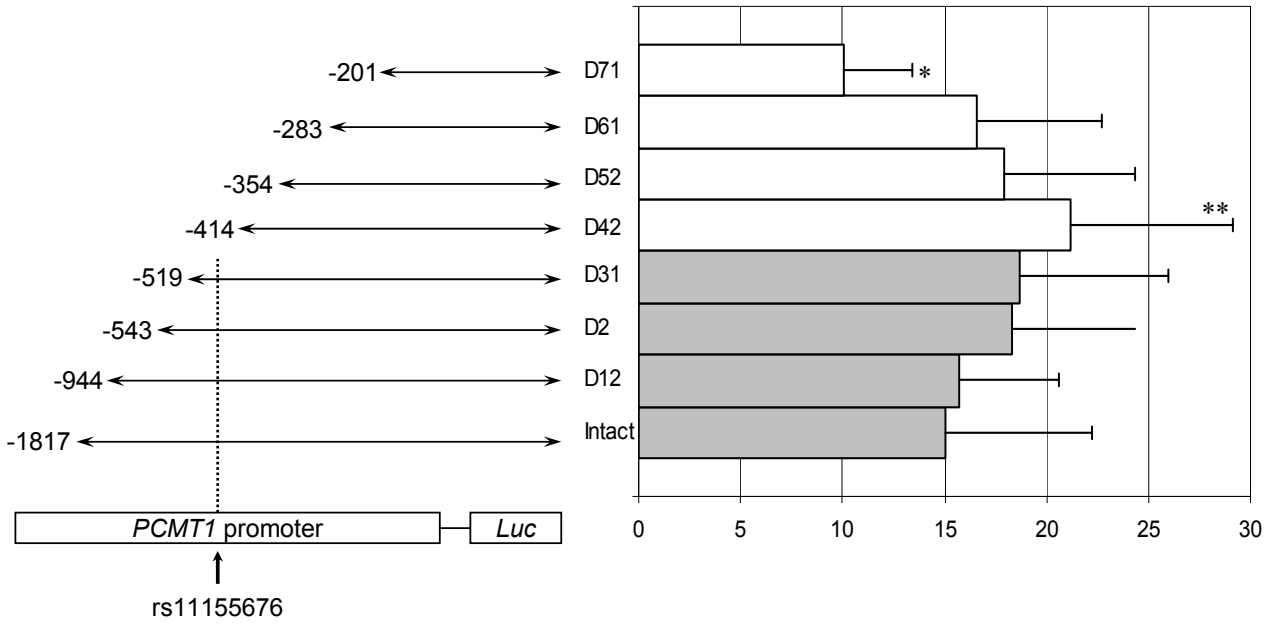

Figure 1. Promoter activity (firefly/renilla luciferase) in INS1e cells transfected with 8 different promoter constructs. * $p < 0.05$ (vs. rest of constructs except Intact and D12). ** $p < 0.05$ (vs. Intact). The diagram to the left shows the length (out of scale) of the 8 promoter constructs used and the position of the polymorphism described.

moter construct was compared with the other ones using a two-sided ANOVA test and Tukey's post-hoc correction.

Results

Genetic analysis

Four variants were identified through initial sequencing of the *PCMT1* gene in the screening samples: one in position 509 upstream of the starting codon (-509 C>G, rs 11155676), one in exon 5 (358 G>A, rs4816) and two in exon 8, within the 3'UTR (845 A>G and 1072 A>T, rs7818 and rs4552, respectively). These four variants were in complete linkage disequilibrium, yielding 2 haplotypes only (Figure 2). After confirming the latter in 96 additional subjects, only one of the variants was genotyped in the rest of the family cohort (genotyping success 99.1%). In the 436 parents genotyped, 35.2% were homozygous for the wildtype haplotype, 15.5% were homozygous for the variant haplotype and 47.7% were heterozygous (expected distribution according to Hardy Weinberg Equilibrium (HWE): 36%, 16% and 48%, respectively, p = 0.98). The variant found in exon 5 (rs4816) leads to an aminoacid substitution (119 Val>Ile) associated with higher enzymatic repair activity [20]. The variant found in the region 5' of the gene changed a putative transcription factor binding site (from AP2 to MDGP).

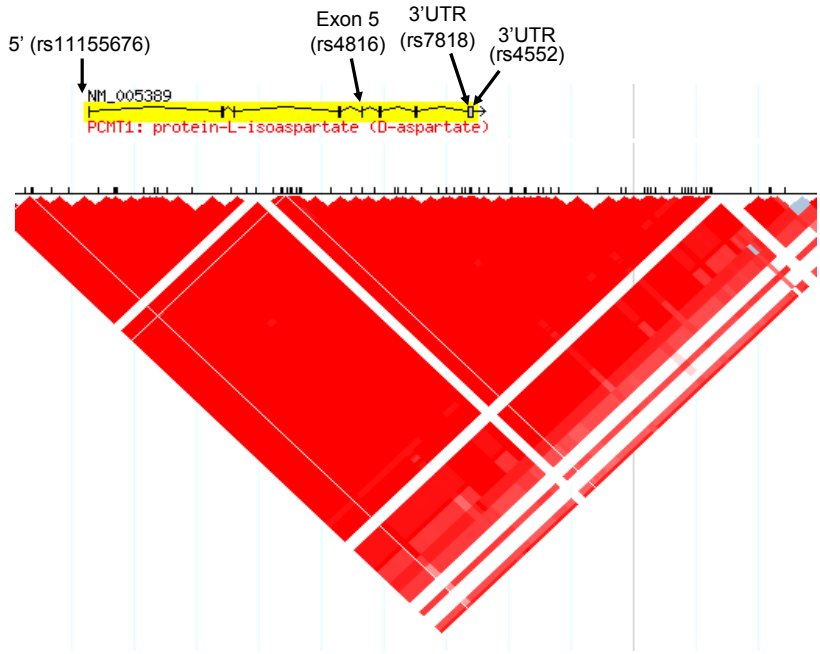

Figure 2. HapMap diagram showing the complete linkage disequilibrium block the *PCMT1* gene is contained in. The arrows indicate the localization of the four polymorphisms found.

The genotyping success for the functional *SUMO4* polymorphism was 97.3%. Of the 428 parents genotyped, 29.8% displayed genotype AA, 47.7% AG and 19.8% GG. (HWE: 30.4%, 49.5% and 20.1%, respectively, p = 0.93).

The variant *PCMT1* haplotype was transmitted to 46% of the affected siblings in the 253 Danish families with T1D (chi-square 1.78, p = 0.18), and to 53% of the non-affected ones (p = 0.099 for transmission to affected vs. non-affected). The functional variant in *SUMO4* was transmitted to 50.9% of the affected offspring (chi-square 0.088, p = 0.767) and to 50.5% of the non-affected offspring. When stratified according to the reported *SUMO4* risk genotypes, there was a trend towards a decreased transmission of the variant *PCMT1* haplotype to the affected offspring, when compared to the non-affected offspring in the families where the proband had 163 GG or AG genotype (high-risk, dominant model) (p = 0.069) (Table 1). Parent homozygocity TDT and sib-TDT did not add significant information (data not shown). The total number of high-risk *PCMT1* and *SUMO4* alleles was similar in affected and unaffected subjects. Regarding *HLA* stratification, there was a trend towards an uneven transmission of the *PCMT1* variant in the non-high risk group only (Table 1).

In the case-control study, rs4816 was also in Hardy-Weinberg equilibrium (using the exact test, p > 0.001). The minor allele (A), associated with higher PIMT activity [20], was present in 40.36% of the affected and in 36.98% of the unaffected subjects (OR 1.15, p = 0.055). For genotype analysis, the dominant model showed a significantly higher frequency of carriers of the A allele among subjects with T1D (490/778 vs. 431/749, p = 0.03).

Promoter activity

Exposure to cytokines significantly increased promoter activity for all the constructs tested (fire-

fly/renilla luciferase ratio 11.6 ± 7.0 for unstimulated vs. 18.6 ± 5.6 for IL1 vs. 16.4 ± 5.3 for IFNγ and 20.1 ± 6.8 for the cytokine mix, $p \leq 0.003$ for all cytokines vs. no treatment). The shorter construct showed significantly lower promoter activity (10.0 ± 3.4, $p \leq 0.05$) than the rest (except for the 2 longest, see Figure 1). However, there was no difference between the constructs adjacent to the previously described variant, namely constructs D31 and 42, see Figure 1 (18.7 ± 7.3 vs. 21.2 ± 8.0, $p = 0.92$).

Table 1. Transmission of the *PCMT1* variant haplotype to affected and non-affected offspring, stratified according to *SUMO4* and *HLA* risk genotypes

Genotype	Transmission to affected offspring, n (%)	p-value	Transmission to non-affected offspring, n (%)
SUMO4			
163 AA or AG	108 (47.6)	NS	75 (56.5)
163 AA	41 (48.8)	NS	20 (50.0)
163 GG or AG	95 (45.5)	NS	79 (55.2)*
163 GG	24 (40.7)	NS	20 (51.3)
HLA			
DR4/DR3	65 (49.6)	NS	46 (55.4)
All other DR	60 (46.2)	NS	40 (48.8)
DR3/3 or DR4/4	17 (48.6)	NS	6 (46.2)
DR3/X or DR4/X	43 (42.6)	NS	32 (49.2)**

Legend: * $p = 0.069$. ** $p = 0.086$ (vs. transmission to affected offspring). NS: not significant.

Discussion

The genetic studies performed in the Danish type 1 diabetes family cohort, showed suggestive, but not conclusive, trends of *PCMT1* haplotypes' involvement in modulating diabetes susceptibility in global and stratified analysis. The low-risk *PCMT1* haplotype is associated with higher enzymatic activity and tends to be preferentially transmitted to the unaffected offspring, especially in certain risk groups defined by other genes (*HLA* and *SUMO4*). However, the non-stratified results of the case-control study pointed in the opposite direction, which weakens our initial conclusion.

The class II MHC plays a principal role in antigen presentation to CD4 cells. Inherited differences in HLA molecules between individuals can determine to which antigens and, in particular, which epitopes an individual can respond. Thus, given a certain *HLA* genotype, the role of other players in the development of the disease may differ. Indeed, specific *HLA* genotypes are the most critical determinants of genetic risk to develop T1D; it is estimated that they account for 40-50% of the genetic risk [14]. Posttranslational modifications can also change a protein's binding affinity for class II MHC molecules [1]. Furthermore, protein deamidation/isomerisation can modify cleavage sites for the enzyme asparaginase endopeptidase, potentially having important consequences for protein processing and antigen presentation by class II MHC molecules [3, 21]. In the present study, *PCMT1* appeared to play a more important role in subjects with any *HLA* genotype except DR3/DR4, i.e. those with lower-risk DR genotypes. On the other hand, *SUMO4*, located within *IDDM5*, less than 1 cM away from *PCMT1*, encodes a protein which binds to IκBα inhibiting NFκB transcriptional activity, and may play a central role in apoptosis. Thus, the genetic interaction between *PCMT1* and both *HLA* and *SUMO4* is biologically plausible. Although the functional variant of *SUMO4* has previously been shown to be associated with T1D [12, 13], neither the present, nor other larger, studies have been able to replicate these findings in Caucasian populations [22]. Nevertheless, as with other minor genes involved in the pathogenesis of T1D, its effect might not be significant on its own, but only through interaction with other genes, as suggested by a recent Swedish study. This work showed a positive association between the functional *SUMO4* variant and T1D in high-risk *HLA* DR3/DR4 subjects [23].

Our finding that *PCMT1* promoter activity is enhanced in INS-1E cells, in response to cytokines, in an *in vitro* model of the disease, argues in favor of a role for PIMT in the pathogenesis of T1D. However, although *in silico* analysis of the polymorphism described in the *PCMT1* promoter region (-509 C>G) revealed a change in a putative transcription factor binding site, no difference was seen in promoter activity between the constructs adjacent to this position. This suggested that eliminating this binding does not have a strong effect on the expression of *PCMT1*. However the effect of creating a new binding site was not addressed by the reporter gene studies.

Some of the proteins recently described as substrates for PIMT in the brain [24, 25] change their expression in pancreas over time in diabetes-prone, but not in diabetes-resistant BB rats [26]. Furthermore, in a cellular model of beta-cell maturation, proteomic studies suggested that posttranslational protein modifications may reflect the acquired sensitivity of the beta cell to cytokines [27]. In the same model, the exposure to cytokines led to a 1.5-fold decrease in *Pcmt1* expression in mature, but not in immature pre-beta-cells [28], suggesting that PIMT response may be part of the specific phenotype making the beta-cell prone to immune-mediated destruction. However, the present study does not support such a decrease, indeed *in vitro PCMT1* promoter activity increased rather than decreased in response to cytokines. Interestingly, *PCMT1* contains several putative targets for miRNAs (miR), which may contribute to the transcriptional regulation of this gene. Of the two polymorphisms identified in exon 8, rs7818 is included within known miRNA targets (for has-miR-361, has-miR-627 and has-miR 589, miRanda miRNA targets, www.t1dbase.org, last accessed on the 23rd July 2008). This suggests that the functional consequences of the 2 different haplotypes described may not only depend on the polymorphism found in exon 5, but also on the regulation of translation mediated by miRNAs.

In conclusion, the present study adds to existing data suggesting a role of *PCMT1* in the pathogenesis of T1D. To date, the following observations support such a role: PIMT is highly expressed in human pancreas, the induction of *PCMT1* delays and reduces diabetes severity in diabetes-prone BB rats, changes in PIMT substrate expression precede the development of diabetes in these animals, *PCMT1* promoter activity is enhanced by *in vitro* exposure of an insulin-producing cell-line to cytokines and, finally, the low-risk *PCMT1* haplotype tends to be preferentially transmitted to non-affected offspring, especially in certain risk groups.

The lack of statistical significance might be due to the size of the population analyzed, which is insufficient to detect a small increase in risk. In addition, the case-control study showed an opposite trend. Power calculations revealed that the present case-control sample had a power of 43% to detect the differences found, with an alpha of 0.05, and that a total of 1898 cases would be needed to reach a power of 80%. Due to the reduced statistical power in the present study, definite conclusions cannot be made. Although a larger sample is needed to confirm the detected association between *PCMT1* and type 1 diabetes, the findings of our present study, together with our previous findings [7], are the first to suggest a possible role of this gene in the development of type 1 diabetes.

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