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Novel minor HLA DR associated antigens in type 1 diabetes

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ABSTRACT

Type 1 diabetes is an autoimmune disease leading to insulin deficiency. Autoantibodies to beta cell proteins are already present in the asymptomatic phase of type 1 diabetes. Recent findings have suggested a number of additional minor autoantigens in patients with type 1 diabetes. We have established luciferase immunoprecipitation systems (LIPS) for anti-MTIF3, anti-PPIL2, anti-NUP50 and anti-MLH1 and analyzed samples from 500 patients with type 1 diabetes at onset of clinical disease and 200 healthy individuals who had a family history of type 1 diabetes but no evidence of beta cell autoantibodies. We show significantly higher frequencies of anti-MTIF3, anti-PPIL2 and anti-MLH1 in recent onset type 1 diabetes patients in comparison to controls. In addition, antibodies to NUP50 were associated with HLA-DRB1*03 and antibodies to MLH1 were associated with HLA-DRB1*04 genotypes.

1. Introduction

Type 1 diabetes is a chronic autoimmune disease targeting the pancreatic beta cells leading to insulin deficiency. Autoantibodies to the beta cell proteins insulin, GAD65, IA-2 and ZnT8 are markers for the diagnosis of pre-symptomatic type 1 diabetes [1].

Recent findings have suggested a number of additional minor autoantigens in patients with type 1 diabetes [2–4]. In addition to the validated finding of antibodies to tetraspanin 7 [4], Bian and colleagues identified NUP50, PPIL2, MTIF3 and MLH1 as possible new target autoantigens in a NAPPA Array based approach [4]. Distinct from the major autoantigens of the pancreatic beta cell, which are found within the secretory granules and vesicles, MLH1, NUP50, and PPIL2 are nucleus associated proteins, and MTIF is a mitochondrial translation initiation factor [5]. Although autoantibodies to minor autoantigens are unlikely to be helpful in disease prediction, they provide insight into mechanisms of immunization and susceptibility.

MLH1 is a DNA mismatch repair enzyme. Mutations in MLH1 cause Lynch syndrome [6,7]. In murine tumor models, inactivation of MLH1 leads to increased generation of neoantigens [8]. MLH1 has been described as an antigen in systemic autoimmune conditions including systemic lupus erythematosus (SLE) and inflammatory myositis (IM) [9,10]. In recent oncologic publications, mismatch repair alterations including MLH1 correlate with altered PD-1/PDL1 signaling [11,12]. The PD-1/PDL1 pathway participates in murine and human type 1 diabetes development [13–15]. NUP50 is part of the nuclear pore complex (NPC) and regulates transport through the NPC. NUP50 also binds to chromatin and regulates transcriptional activity [16]. MTIF3 is a mitochondrial translation initiation factor and is implicated in obesity and Parkinson disease [17–22]. PPIL2 is a member of the cyclophilin family, but is unlikely to have PPIAse activity under physiological conditions [23]. It may be involved in intracellular transport [24].

Abbreviations: IM, Inflammatory myositis; MLH1, MutL homolog; MTIF3, Mitochondrial translational initiation factor 3; NAPPA Array, Nucleic acid programmable protein arrays; NUP50, Nucleoporin 50; PD-1, Programmed cell death protein 1; PDL1, Programmed cell death 1 ligand 1; PPIL2, Peptidyl-prolyl cis-trans isomerase-like 2; SLE, Systemic lupus erythematosus

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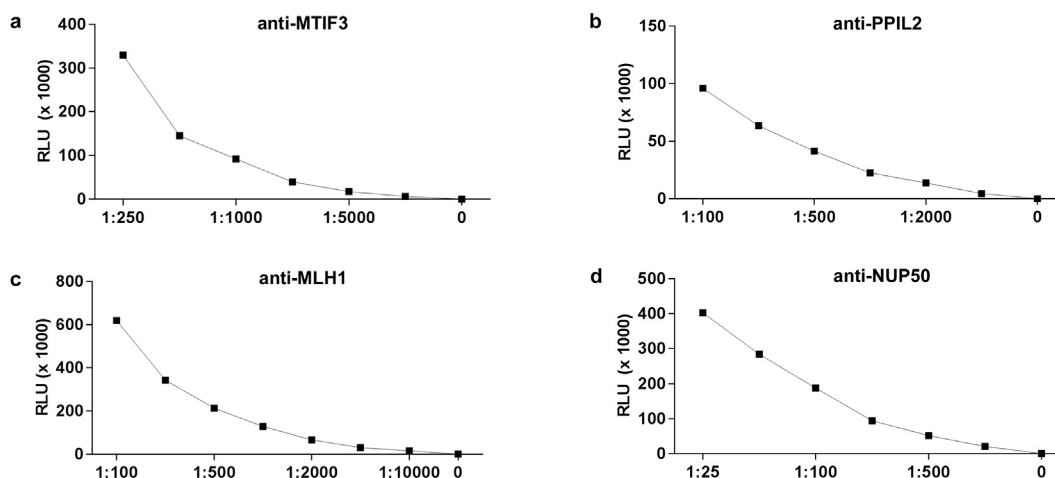

Fig. 1. Validation of anti-MTIF3, anti-PPIL2, anti-MLH1 and anti-NUP50 LIPS. Commercial antibodies were diluted in known beta cell autoantibody negative serum and analyzed with the LIPS using PPIL2 (a), MTIF3 (b), MLH1 (c) and NUP50 as autoantigens (d).

In order to determine whether these nucleus- and mitochondria-associated proteins are relevant autoantigens in type 1 diabetes, we developed luciferase immunoprecipitation systems (LIPS) assays for the detection of anti-NUP50, anti-MTIF3, anti-MLH1 and anti-PPIL2 autoantibodies and tested sera from recent onset type 1 diabetes patients and known beta cell autoantibody negative first-degree relatives of type 1 diabetes patients. The results confirm that PPIL2, MTIF3 and MLH1 are novel minor autoantigens in type 1 diabetes with HLA class II allele association. The presence of generalized autoimmunity in type 1 diabetes is consistent with the partially shared regions of genetic susceptibility between type 1 diabetes and other autoimmune diseases.

2. Material and methods

2.1. Participants

Sera from the DiMelli cohort of patients with new onset type 1 diabetes [25] and the TeenDiab cohort of prospectively followed first degree relatives of type 1 diabetes patients were selected for analyses [26,27]. The sample set included sera from 500 patients with recent onset type 1 diabetes (age range, 1–19 years, median age 10 years, 222 females, median days since diagnosis 9) and 200 samples from islet autoantibody negative first-degree relatives as controls (age range 6–18 years, median age, 11 years, 90 females). An extended sample set of patients who had HLA DR allele typing information included sera from an additional 751 patients in the DiMelli recent onset type 1 diabetes cohort for the measurement of antibodies to NUP50, 740 of which could also be measured for antibodies to MLH1. A summary of the samples used in the study is shown in Supplementary Table 1. The ethical committees of Bavaria or the Ludwig Maximilian University Munich approved the studies (protocol numbers 08043 and 2149/08), which were carried out in accordance with the Declaration of Helsinki, as revised in 2000. Informed, written consent was obtained from patients or parents of participants.

2.2. Cloning and expression of antigens

cDNA from human peripheral mononuclear cells was used to clone full length *NUP50* and *MTIF3* into pCMV6-AC-IRES-GFP-Puro (Origene, MD, USA) containing NanoLuc® Luciferase. cDNA from human islets was used to clone *MLH1* into the expression vector pTNT (Promega, Mannheim, Germany) containing NanoLuc® Luciferase using restriction site cloning. *PPIL2* was synthesized and inserted into pG9m-1 (Baseclear, Switzerland) and then subcloned into pCMV-Nluc-IRES-GFP-Puro via *Bam*HI and *Not*I restriction sites (Thermo Fisher Scientifc, MA, USA). The final constructs encoded N-terminal NanoLuc® Luciferase-fusion proteins. HEK293 cells were transfected with the pCMV- driven plasmids and cell pellets were lysed in 1% Triton X-100 lysis buffer (100 μl/10^6 cells) for 30 min at 4 °C. After centrifugation (10 min at 13000 rpm 4 °C) the supernatant was collected and used in immunoassays. For pTNT vectors, the TNT® Quick Coupled Transcription/Translation System (Promega, Mannheim, Germany) was used according to the manufacturer's instructions to generate the antigens.

2.3. Luciferase ImmunoPrecipitation System (LIPS) assays

LIPS were performed as previously described [2]. Briefly, serum (2 μl) was added to duplicate wells of a 96-well microfiltration plate (Merck Millipore, Darmstadt, Germany) containing 23 μl of assay buffer and 5 million counts per second of NanoLuc-tagged antigen. After 2 h incubation (dark, RT), protein-A Sepharose (GE Healthcare, Freiburg, Germany) equivalent to 1.5 mg per well, pre-swollen in assay buffer containing 0.1% BSA low IgG (Life Technologies, Darmstadt, Germany) was added. Plates were shaken (300 rpm/min) at 4 °C for 1 h followed by washing. Activity was measured after the addition of NanoLuc Glo luciferase substrate (Promega, WI, USA). All test samples were received and measured as coded samples with decoding performed centrally after all measurements were completed. Commercially available antibodies diluted in antibody negative serum were used as calibrators and for assay validation (anti-MLH1: Clone G168–728, BD Biosciences; NJ, USA; anti-MTIF3: Cat# 14219-1-AP, Proteintech, Hubei, R.P.C.; anti-NUP50: Cat# NB100–93324, Novus Biologicals, CO, USA; anti-PPIL2: Cat# 104491NovoPro, Shanghai, R.P.C.).

2.4. Antinuclear antibodies (ANA)

Sera were tested at 1:10 dilution in PBS, applied onto slides with Hep-2 cells (Antibodies Incorporated, CA, USA), washed in assay buffer, incubated with FITC-conjugated anti-human gamma globulin antibodies (Antibodies Incorporated; CA, USA) and after a second wash, examined using a DMi8 fluorescence microscope (Leica, Wetzlar, Germany). Images were processed using ImageJ (NIH; MD, USA).

2.5. Statistics

Graphical presentation of the data, correlation analyses, and comparisons between groups were made with GraphPad Prism software (GraphPad Software, Inc., CA, USA). Fisher's test was used for comparisons of antibody positives between groups (T1D vs control; DR3 vs non DR3

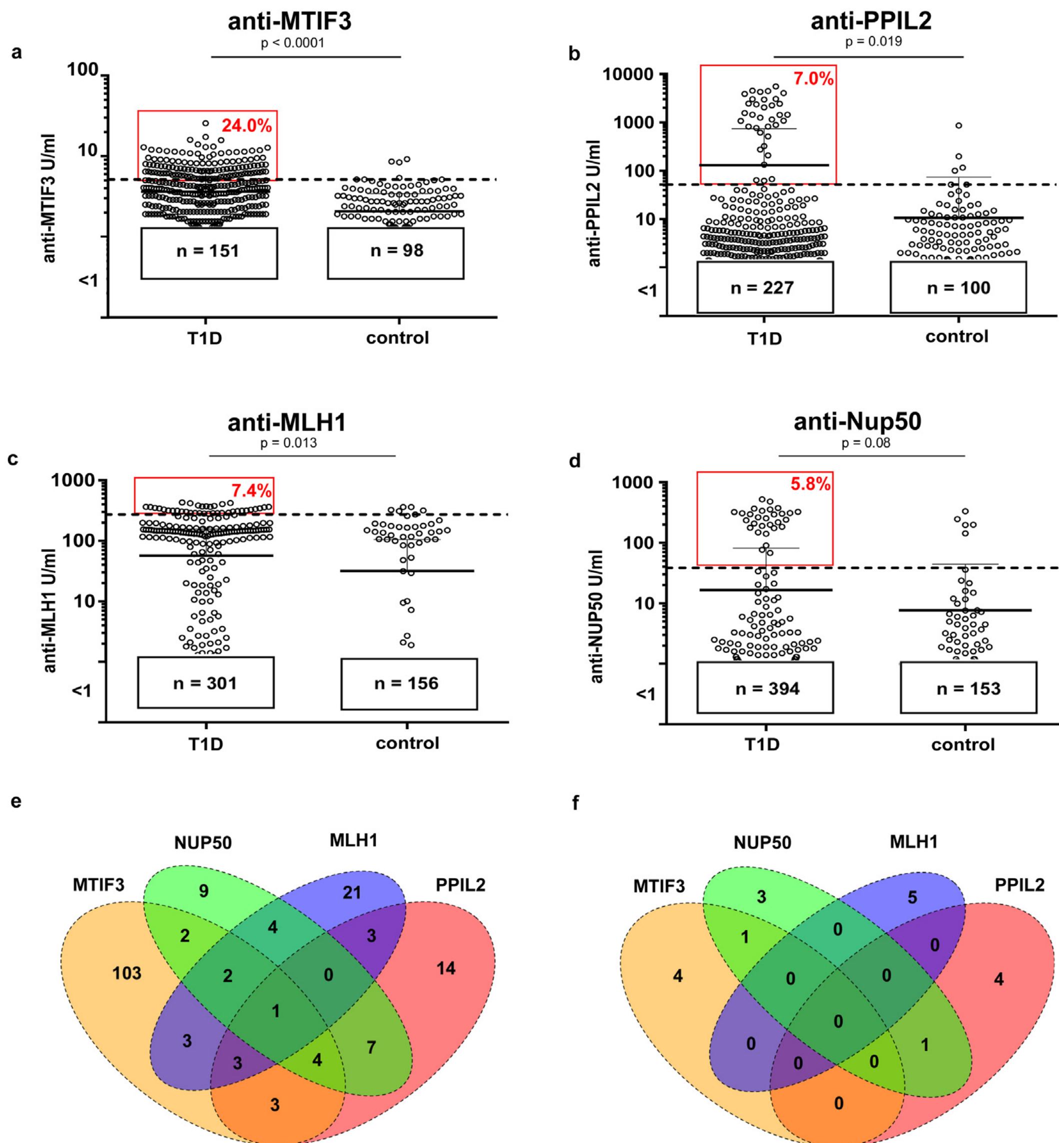

Fig. 2. Frequency of novel autoantibodies in patients with recent onset type 1 diabetes Autoantibodies to MTIF3 (a), PPIL2 (b), MLH1 (c) and NUP50 (d) autoantibodies were analyzed in patients with recent onset type 1 diabetes (T1D; n = 500) and first degree relatives of type 1 diabetes patients (n = 200). Percentages of positive patients (T1D) is indicated in red font. Cut-offs (dashed line) represent the 97.5th centile of healthy controls. Antibody distribution is shown as Venn diagrams for T1D (e) and controls (f; MTIF3 (orange), NUP50 (green), MLH1 (blue), PPIL2 (red)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and DR4 vs non DR4). Venn diagrams were made using "Venn.Diagram" (https://CRAN.R-project.org/package=VennDiagram; Hanbo Chen) with R studio (RStudio Inc., Boston, MA).

3. Results

Methods were established and validated using commercial anti-MLH1, anti-NUP50, anti-MTIF3 and anti-PPIL2 antibodies diluted in human serum (Fig. 1). Titration curves were obtained for each of the antigens, allowing subsequent measurement of antibodies in serum samples.

Using a threshold at the 97.5th centile of the 200 control samples, anti-MTIF3-, anti-PPIL2-, anti-MLH1- and anti-NUP50 antibodies were detected in 121 (24%; $p < 0.0001$), 35 (7%; $p = 0.019$), 37 (7.4%; $p = 0.013$) and in 29 (5.8%; $p = 0.08$) of the 500 patients with recent onset type 1 diabetes, respectively (Fig. 2a-d). Within the patients, there was an association between anti-MLH1, anti-NUP50, and anti-PPIL2 (Fig. 2e; Supplementary Table 2). Anti-MLH1 antibodies were observed more frequently in patients who were positive for anti-NUP50 or anti-PPIL2 (13/52, 25%) than in patients who were anti-MTIF3 positive (9/121, 7.4%; $p = 0.0026$) and in patients who were negative for anti-MTIF3, anti-NUP50 and anti-PPIL2 antibodies (21/342, 6.1%; $p = 0.0001$). A similar association was observed for anti-PPIL2 antibodies (18/59, 30.5%, vs 11/121, 9.1%; $p = 0.0004$ in anti.MTIF3 positive patients; and 14/335, 4.2%; $p < 0.0001$ in anti-MTIF3, anti-NUP50 and anti-MLH1 negative patients), and for anti-NUP50 antibodies (18/65, 27.7% vs 9/121, 7.4%; $p = 0.0003$ in in anti.MTIF3 positive patients; and 9/330; 2.7%, $p = 0.0001$ in anti-MTIF3, anti-PPIL2 and anti-MLH1 negative patients). No associations were observed in the control group. Antibodies to two or more of the 4 antibodies were observed in 32 (6.4%) of 500 patients and in 2 (1%) of 200 controls ($p = 0.0025$). No correlations were observed between the autoantibodies and autoantibodies to GAD, IA-2 or ZnT8 (data not shown).

A number of control individuals had high titers of antibodies to the novel autoantigens. Since the controls were relatives of patients and therefore enriched in HLA risk alleles, we examined whether there was

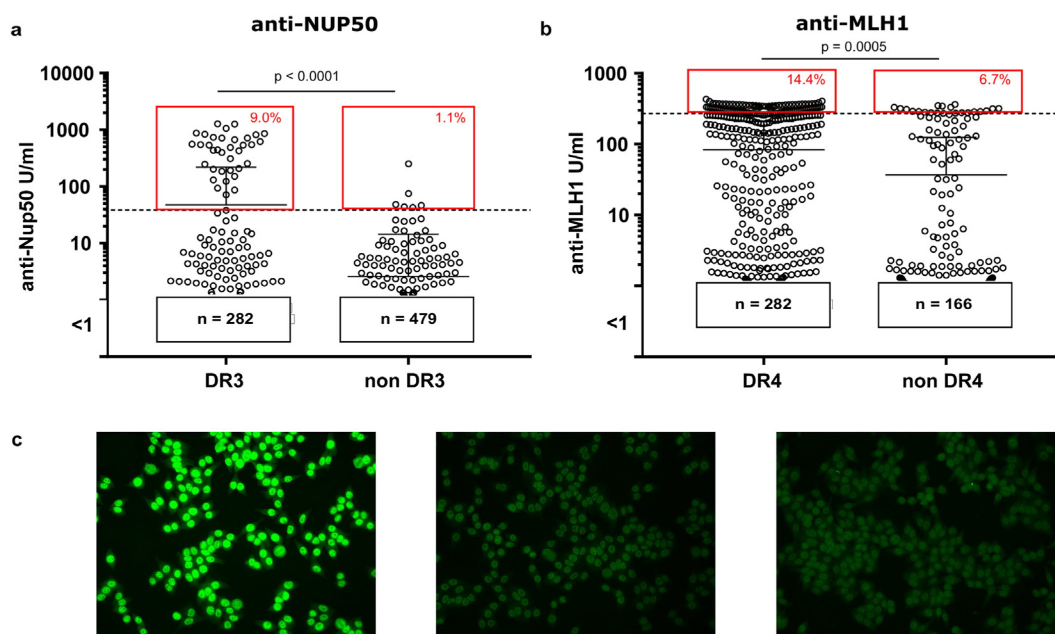

Fig. 3. HLA DR association of anti-NUP50 and anti-MLH1 and antinuclear antibodies. Anti-NUP50 antibodies are more frequent in type 1 diabetes patients with DR3 genotype ($p < 0.0001$, a) and anti-MLH1 autoantibodies in DR4 positive type 1 diabetes patients ($p = 0.0005$, b). Anti-nuclear antibody by immunofluorescence on Hep2 cells (c) is shown for an anti-Nup50 and anti-MLH1 positive serum (left panel), an anti-PPIL2 positive serum (middle panel), and a negative control (right panel)

a relationship between the presence of autoantibodies and HLA DR3 or HLA DR4. Information on the HLA DR alleles was available in 204 patients and 188 controls from the first sample set. Among the controls with HLA typing, the majority (4/5) with NUP50 antibody titers above the 97.5th centile had HLA DR3, and the majority (4/5) of controls with MLH1 antibody titers above the 97.5th centile had the HLA DR4 allele. We, therefore, examined an extended set of patients who had HLA DR allele information. Antibodies to NUP50 were more frequently detected in the patients who had HLA DR3 (35/389, 9.0%) as compared to patients who did not have HLA DR3 (6/566, 1.1%; $p < 0.0001$). Antibodies to MLH1 were more frequently detected within patients who had HLA DR4 (93/645, 14.4%) as compared with patients who did not have HLA DR4 (20/299, 6.7%; $p = 0.0005$; Fig. 3a, b).

Since PPIL2, MLH1 and NUP50 are nucleus-associated proteins, we further assessed positivity using indirect immunofluorescence on Hep2 cells. Both samples with the highest LIPS assay antibody titers against MLH1 and NUP50 were found positive on Hep2 cells. No nuclear staining was observed for the autoantibody negative controls (n = 3) and the serum with the highest PPIL2 titer (Fig. 3c).

4. Discussion

MTIF-3, MLH1 and PPIL2 were confirmed as minor autoantigens in type 1 diabetes. The frequency of antibodies to these proteins and of antibodies to NUP50 is relatively low and measurement of these antibodies is unlikely to provide benefit over existing strategies to identify individuals at risk of type 1 diabetes. Nevertheless, autoimmunity against nuclear and mitochondrial proteins is not typically described in type 1 diabetes and may indicate a distinct immunization process in a subgroup of patients or a manifestation that is linked to shared genetic susceptibility between autoimmune diseases.

Our study confirms the initial findings obtained through array-based screening. Thus, the array approach appears to be a robust strategy to identify antibody targets. It is unclear, however, whether the increased frequency of the antibodies observed in patients is relevant to disease or is a secondary association with HLA and other susceptibility genes. For two of the antigens, we found associations between the antibodies and HLA class II alleles that are frequent in patients with type 1 diabetes. We have not tested samples prior to diabetes onset and, therefore, do not know how early they may appear.

Of the antigens tested, antibodies to mitochondrial protein MTIF3 were the most frequent and appeared to be of the antibodies against the three nuclear proteins, which were associated with each other in the patient cohort. Antibodies to MTIF3 have not been identified in other diseases, including primary biliary cholangitis, where antibodies to mitochondrial proteins are a characteristic finding [28]. Antibodies to MLH1 have been previously described in SLE patients and in systemic autoimmune diseases such as myositis [9]. Neither of these diseases are associated with HLA DR4. MLH1 inhibition increases neoantigen generation in tumors [8]. It is unknown whether autoantibodies inhibit MLH1 function, but it is possible that their presence could have a similar effect and exacerbate autoimmunity to other antigens. Immunization to nuclear and mitochondrial antigens in the systemic autoimmune diseases is postulated to arise from cell death and autophagy [29]. Neutrophil extracellular traps (NET) formation has also been suggested to play a role in nuclear antigen immunization [30]. Of interest, neutrophils have been suggested to be involved in type 1 diabetes pathogenesis [31], and a neutrophil signature is described in the proteome of $CD4^+$ T cells in recent onset type 1 diabetes patients [32].

5. Conclusion

We show that MTIF3, PPIL2, and MLH1 are minor autoantigens in recent onset type 1 diabetes. Autoimmunity against ubiquitously expressed proteins in type 1 diabetes is consistent with a generalized genetic susceptibility to autoimmunity and suggests that there is also a systemic autoimmune component to the disease process in some patients.

Declaration of interest

None.

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Appendix A. Supplementary data

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