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A study of type-1 diabetes associated autoantibodies in schizophrenia

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Abstract

Epidemiological studies revealed an association between type-1 diabetes (T1D) and schizophrenia but the findings reported to date have been controversial. To clarify the inconsistency across studies, T1D-associated autoantibodies were examined in plasma samples collected from 272 patients with schizophrenia and 276 control subjects. An in-house enzyme-linked immunosorbent assay (ELISA) was developed using three linear peptide antigens, one of which was derived from glutamic acid decarboxylase (GAD) and two were derived from insulinoma-associated antigen 2 (IA2). Mann-Whitney U test showed a significant decrease in the levels of plasma IgG against the IA2b antigen in schizophrenia patients as compared to control subjects ($Z= -3.54$, $p=0.0007$), while no significant difference was found between these two groups either in anti-IA2a IgG levels ($Z= -1.62$, $p=0.105$) or in anti-GAD IgG levels ($Z= -1.63$, $p=0.104$). Linear regression analysis indicated no association of antipsychotic medication with the levels of plasma IgG against IA2a, IA2b or GAD, while the levels of plasma IgG for these 3 peptide antigens were significantly correlated with each other. Binary logistic regression showed that neither the DQ2.5 variant nor the DQ8 variant was associated with circulating levels of 3 T1D-associated autoantibodies in both the patient group and the control group. The coefficient of variation was 10.7% for anti-IA2a IgG assay, 10.1% for anti-IA2b IgG assay and 10.7% for anti-GAD IgG assay. The present work suggests that T1D-associated antibodies are unlikely to confer risk of schizophrenia and that the in-house ELISA developed with linear peptide antigens is highly reproducible.

Keywords: Autoantibody; ELISA; schizophrenia; type-1 diabetes

1. Introduction

Schizophrenia is a severe psychiatric disorder with an incidence rate of 0.7% in the population worldwide (Saha et al., 2005). It is a complex heterogeneous disorder that presents clinically with positive symptoms (such as hallucinations and delusions), negative symptoms (such as apathy and social withdrawal) and cognitive impairments (such as working memory loss). The etiology of schizophrenia is complex and as yet is still unclear, although it is known to be a highly heritable disorder with inheritability of up to 81% (Sullivan et al., 2003). A multi-stage schizophrenia genome-wide association (GWA) study confirmed that 108 loci in the human genome were involved in genetic risk of the disease (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), suggesting that schizophrenia is a polygenic disorder with a heterogeneous pattern. This GWA study also showed that the strongest association signal was identified in the human major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) locus in the short arm of chromosome 6 ($p=3.48 \times 10^{-31}$); enrichment analysis revealed that most of these susceptibility genes were highly expressed in both human brain and B-lymphocytes, indicating an important role in immunity.

The HLA region is highly polymorphic and contains more than 100 genes essential for the regulation of immune function; it is also involved in predisposition to a number of human diseases such as inflammatory diseases and autoimmune disorders. Type-1 diabetes (T1D) is an autoimmune disease mainly caused by autoimmune destruction of pancreatic ß-cells, leading to the failure of producing insulin for glucose metabolism. Several epidemiological studies suggested that T1D was associated with schizophrenia but the findings reported to date have been controversial. Eaton et al. (2006) reported a lower prevalence of T1D in patients with schizophrenia than control subjects; Juvonen et al. (2007) reported a decreased incidence of schizophrenia in patients with T1D. Conversely, a couple of studies reported an increase in T1D prevalence among patients with schizophrenia, with a relative risk/odds ratio of 1.27 and 1.54, respectively (Benros et al., 2011; Chen et al., 2012). In addition, the HLA class II (HLA-II) alleles coding for DQ8 and DQ2.5 molecules account for up to 50% of genetic risk of T1D (Todd, 2010; Steck and Rewers, 2011) and over 60%

T1D patients carry DQ2/DQ8 genotypes (Eringsmark Regnéll and Lernmark, 2012). However, GWA studies have confirmed that the frequencies of DQA1*0501 and DQB1*0201 variants coding for DQ2.5 molecule are significantly lower in patients with schizophrenia than control subjects (International Schizophrenia Consortium, 2009; Sekar et al., 2016), and in a recent study we have also found no association of the DQ8 variant with schizophrenia in a Scottish population (unpublished data). Therefore, schizophrenia and T1D are unlikely to share the same genetic risk determined at the HLA-II locus.

To clarify the inconsistency between epidemiological studies, it is useful to examine the prevalence of T1D-associated autoantibodies in schizophrenia. More than five distinct T1D-associated autoantigens have been identified for autoimmune responses in T1D, including insulin, glutamic acid decarboxylase (GAD), insulinoma-associated antigen 2 (IA2), zinc transporter 8 (ZnT8) and islet cell antigens (ICAs). Both GAD and IA2 are involved in function of the central nervous system and the neuroendocrine system (Nishida et al., 2009; Davis et al., 2016). GAD is an enzyme that catalyzes the decarboxylation of glutamate to ϒ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in adult mammalian brain, which plays a major role in the development of normal brain and has been strongly implicated in the pathobiology of schizophrenia (Wassef et al., 2003; Straub et al., 2007; Taylor and Tso, 2015). IA2 has been found to be highly expressed in the pituitary gland (Nishida et al., 2009). A few studies have been performed to examine autoantibodies against GAD and IA2 in schizophrenia but failed to show a significant change (Cohen et al., 2005; Yarlagadda et al., 2008; Ezeoke et al., 2013). Because the sample size used in the above studies was rather small, it is particularly important to replicate such initial work in a large sample size. This study was thus undertaken to develop an in-house enzyme-linked immunosorbent assay (ELISA) using linear peptide antigens derived from GAD and IA2, and to analyse the levels of autoantibodies against these peptide antigens in plasma samples from schizophrenia patients and control subjects.

2. Materials and Methods

2.1 Subjects

A total of 548 archived plasma samples collected from patients with schizophrenia (n=272, 192 males and 80 females, mean age 44.7 ±12.1 years) and control subjects (n = 276, 208 males and 68 females, mean age 41.9 ± 13.0 years), were used to examine circulating levels of IgG antibodies against GAD and IA2. These case-control samples were collected through the University of Aberdeen in the period between 2003 and 2008. All the subjects were classified as British Caucasian, including English, Scottish, Welsh and Irish individuals. All patients were diagnosed as having schizophrenia based on the DSM-IV criteria and control subjects were screened for psychiatric disorders as mentioned in previous GWA study (International Schizophrenia Consortium, 2009). Antipsychotic drugs used by schizophrenia patients at the time of sampling are listed in Table 2; of these 272 patients, 171 were taking a single antipsychotic drug, 31 were taking more than one drug and 70 failed to give medication details. All subjects gave written informed consent to giving blood samples for study of the pathogenesis of schizophrenia. This study was approved by a local ethics committee and conformed to the Declaration of Helsinki.

2.2 Detection of plasma IgG

Three linear peptide antigens were applied to develop an in-house ELISA, one derived from GAD and two derived from IA2; their amino acid sequences are given in Table 1, of which IA2a was designed based on the computational prediction of HLA-II epitopes (Söllner et al., 2010; Wang et al., 2010), IA2b based on epitope LGPEGAHGDTTFEYQDL sequence that was confirmed to be involved in the autoimmune responses in patients with T1D (http://www.pepperprint.com/), and GAD based on the epitope information for human autoimmune disease in the Immune Epitope Database (http://www.iedb.org/). In brief, Maleimide-activated plates (Cat. 15150, Thermo Scientific, Edinburgh, UK) were coated based on the Manufacturer's instruction. The antigen-coated plate was washed twice with 200µl Wash Buffer that was phosphate-buffered saline (PBS) (P4417, Sigma-Aldrich, Ayrshire, UK) containing 0.05% Tween-20; 50µl plasma sample diluted 1:200 in Assay

Buffer that was PBS containing 0.5% bovine serum albumin (BSA) was then added to each sample well; 50µl Assay Buffer was added to each negative control (NC) well and 50µl positive control (PC) sample was added to each PC well. Following incubation at room temperature for 1.5 hours, the plate was washed three times with 200µl Wash Buffer and 50µl peroxidase-conjugated goat anti-human IgG antibody (ab98567, Abcam, Cambridge, UK) diluted 1:30000 in Assay Buffer was added to each well. After incubation at room temperature for 1 hour, colour development was initiated by adding 50µl Stabilized Chromogen (SB02, Life Technologies, Warrington, UK) and terminated after 20 min by adding 25µl Stop Solution (SS04, Life Technologies, UK). The measurement of optical density (OD) was completed on a microplate reader within 10 min at 450nm with a reference wavelength of 620nm. All the samples were tested in duplicate and the specific binding ratio (SBR) was used to represent the relative levels of plasma IgG antibodies. Calculation of SBR is as follows:

$$SBR = (OD_{Sample} - OD_{NC}) / (OD_{PC} - OD_{NC})$$

To minimize an intra-assay deviation, the ratio of the difference between duplicated OD values of each sample to their sum was used to assess the precision for the in-house ELISA antibody test. If the ratio was found to be >10%, the test of this sample was treated as invalid and was not used for data analysis.

2.3. Genotyping of the DQ2/DQ8 variants

HLA-tagging single nucleotide polymorphisms (SNPs) rs2187668 and rs7454108 were used to genotype the alleles coding for DQ2.5 and DQ8 molecules using a TaqMan protocol, in which rs2187668 minor allele "A" represents the DQA1*0501~DQB1*0201 haplotype (DQ2.5) and rs7454108 minor allele "G" represents the DQA1*0301~DQB1*0302 haplotype (DQ8) in the European Caucasian population (Monsuur et al., 2008). TaqMan genotyping reagents were supplied by Applied Biosystem (Paisley, UK). The OneStep real-time PCR system (Applied Biosystems) was used to amplify DNA samples for genotyping of rs2187668 and rs7454108; genotype calls were made manually based on the distribution of three clusters resulting from plotting the amplification of allele 1 versus allele 2.

2.4 Data analysis

Kolmogorov-Smirnov test was applied to test the distribution of antibodies levels in both the patient group and the control group. Because circulating levels of IgG antibodies against 3 antigens all failed to show a normal distribution (Table 3), Mann-Whitney U test was then applied to examine the difference in SBR between the patient group and the control group. The p-value of <0.017 was set for rejection of null hypothesis as 3 antigens were tested in this study. The coefficient of variation (CV) was used to represent an inter-assay deviation estimated using pooled plasma samples, namely quality control (QC) sample, which were randomly collected from >20 healthy subjects and tested on every 96-well plate. Linear regression was applied to examine which antipsychotic drugs might affect the secretion of plasma antibodies specific for the above 3 antigens. In such analysis, the antibody levels were used as a dependent variable, and medication, age and sex were used as the independent variables. Binary logistic regression was applied to examine if DQ2.5 and DQ8 were associated with altered levels of plasma IgG antibodies against these 3 antigens.

3. Results

The CV estimated based on the SBR from the QC sample was 10.7% for anti-IA2a IgG assay, 10.1% for anti-IA2b IgG assay and 10.7% for anti-GAD IgG assay (Table 4), suggesting that the in-house ELISA developed with linear peptide antigens was highly reproducible.

Mann-Whitney U test revealed a significant decrease in plasma anti-IA2b IgG levels in the patient group when compared to the control group (Z= -3.54, p=0.0007), and male patients mainly contributed to the decrease in anti-IA2b IgG levels (Table 5); there was no significant difference found between these two groups either in anti-IA2a levels (Z= -1.62, p=0.105) or in anti-GAD IgG levels (Z= -1.63, p=0.104). Linear regression analysis showed no association of antipsychotic medication with the levels of plasma IgG against IA2a, IA2b or GAD (Table 6). Binary logistic regression showed that neither the DQ2.5 variant nor the DQ8 variant was associated with circulating

levels of IgG antibodies against these 3 antigens in both the patient group and control group (Tables 7 and 8).

As shown in Figure 1, correlation analysis demonstrated that circulating levels of IgG antibodies against IA2a, IA2b and GAD were significantly correlated with each other, including anti-IA2a IgG vs anti-IA2b IgG ($r=0.591$, $df=547$, $p<0.001$), anti-IA2a IgG vs anti-GAD IgG ($r=0.480$, $df=547$, $p<0.001$) and anti-IA2b IgG vs anti-GAD IgG ($r=0.663$, $df=547$, $p<0.001$).

4. Discussion

Epidemiological studies that look at the link between T1D and schizophrenia so far have been inconsistent. The question that the epidemiological data pose is whether T1D is a risk factor for schizophrenia or is protective against the development of schizophrenia. The results from this study suggest that T1D-associated antibodies are unlikely to confer a risk of developing schizophrenia in the Caucasian population. Of these three peptide antigens derived from GAD and IA2 (Table 1), two failed to show immunogenic stimulation of T1D-associated antibody secretion, and the IA2b antigen might induce immune tolerance to its self in patients with schizophrenia (Table 5). While the IA2b antigen is not a HLA-DQ2/DQ8 restricted antigen based on the computational prediction of HLA-II epitopes (Söllner et al., 2010; Wang et al., 2010), it carries the LGPEGAHGDTTFEYQDL sequence that has been confirmed to be involved in the autoimmune responses in patients with T1D (http://www.pepperprint.com/). The present work also showed that neither the DQ2.5 variant nor the DQ8 variant was associated with anti-IA2 and anti-GAD IgG antibodies (Tables 7 and 8). These observations suggest that both DQ8 and DQ2.5 molecules may present peptide antigens from the environment rather than T1D-associated autoantigens (Eringsmark Regnéll and Lernmark, 2012). It has been demonstrated that early exposure to complex dietary proteins like cow's milk could increase the risk of beta-cell autoimmunity in the DQ2.5/DQ8 carriers and milk formula of casein hydrolysate could reduce such a risk (Knip et al., 2010).

It is worth noting that family history of T1D has been consistently reported to be a risk factor for schizophrenia. Wright and colleagues reported a significant excess of T1D in the first-degree relatives of schizophrenia patients, especially in mothers (Wright et al., 1996); Eaton et al. (2006) showed an incidence rate ratio of 1.5 for T1D prevalence in parents of schizophrenia patients, relative to parents of comparison subjects; Benros et al. (2012) demonstrated that the individuals with T1D family history had relative risk of 1.3 to develop schizophrenia. Because the role of T1D family history in enhancing risk of schizophrenia may not be related to the HLA-DQ2.5/DQ8 genotypes based on genetic analysis of schizophrenia as mentioned above, maternal issue should be taken into account. Analysis of IgG antibodies in cord blood samples revealed that maternal IgG antibodies to infectious agents and wheat gluten could confer risk of non-affective psychoses such as schizophrenia and autism (Blomström et al., 2012; Karlsson et al., 2012) as maternal IgG can cross the placenta. Accordingly, we hypothesize that the mechanism behind the involvement of T1D family history in schizophrenia risk may be due to maternal T1D-associated IgG autoantibodies. It has been found that approximately 3% of infants of non-diabetes mothers are positive for T1D-associated autoantibodies in their cord blood samples (Hämäläinen et al., 2002). Therefore, there is an urgent need to establish the link between T1D-associated autoantibodies and schizophrenia.

While male patients significantly contributed to decreased anti-IA2b IgG levels, gender does not appear to serve as a confounding factor for antibody production as female patients also showed a decrease in anti-IA2b IgG levels that did not achieve statistical significance due to small sample size (Table 5). Linear regression analysis showed no association of antipsychotic medication with the levels of plasma IgG against IA2a, IA2b or GAD, but this analysis is limited by the absence of drug-free or naïve patients and incomplete medication histories. In conclusion, T1D may have a complicated relationship with schizophrenia and it is unlikely to confer risk of schizophrenia although maternal T1D-associated autoantibodies may be a risk factor for the disease. In addition, the in-house ELISA developed with linear peptide antigens in this study is highly reproducible and may be useful for large-scale screening of T1D-associated autoantibodies in the population.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Philomena Hallford mainly carried out laboratory work and drafting the manuscript.

David St Clair was mainly responsible for identification of patients, healthy controls and sample collection.

Lorna Halley and Colette J. Mustard were involved in sorting of samples and database as well as genotyping of the HLA-II genes.

Jun Wei conceived of this study, supervised laboratory work and data analysis, and corrected the manuscript.

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Table 1. Sequence of peptide antigens used for in-house ELISA

Antigen	**Sequence**
IA2a	LVRSKDQFEFALTAVAEEVNAILKALPQCH
IA2b	RLAALGPEGAHGDTTFEYQDLCRQH
GAD	CRPTLAFLQDVMNILLQYVVKSFDRSTK

Table 2. Antipsychotic drugs used by schizophrenia patients at the time of sampling

Medication	Patient (n)	Percentage of all cases (%)	Male (n)	Age (years)*
Amisulpride	9	3.3	6	38.6 ±11.1
Clozapine	48	17.6	36	34.1 ±9.9
Haloperidol	1	0.04	0	46.0
Olanzapine	32	11.8	22	41.7±13.8
Phenothiazine	15	5.5	8	50.1±9.1
Quetiapine	8	2.9	7	33.0±12.0
Risperidone	18	6.6	11	53.3±13.0
Sulpiride	3	1.1	2	52.4±17.2
Thioxanthine	37	13.6	24	46.2±11.0
More than 1 drug	31	11.4	23	40.2±11.6
Unknown	70	25.7	52	42.0±12.4

*Mean±SD

Table 3. Kolmogorov-Smirnov test for normal distribution of circulating antibody levels

Antibody	Skewness	Kurtosis	D statistic	p
Patient				
IA2a	1.3	2.18	0.12	<0.0001
IA2b	1.7	5.70	0.11	<0.0001
GAD	1.1	1.68	0.11	<0.0001
Control				
IA2a	1.9	4.73	0.14	<0.0001
IA2b	1.3	2.02	0.12	<0.0001
GAD	1.7	4.65	0.10	<0.0001

Table 4. Inter-assay deviation between ELISA-testing plates

Antigen	**Number of plates**	**Mean**±SD*	**CV (%)**
IA2a	41	0.794±0.085	10.7
IA2b	41	0.636±0.064	10.1
GAD	43	0.747±0.080	10.7

* Antibody levels were expressed in SBR

Table 5. Comparison of circulating levels of IgG against IA2 and GAD peptide antigens in plasma samples

Antigen	**Schizophrenia Mean rank (n)**	**Control Mean rank (n)**	**Z**	**p ***
IA2a				
Male	192.7 (192)	207.8 (208)	-1.31	0.192
Female	71.6 (80)	78.3 (68)	-1.00	0.317
Combined	263.5 (272)	285.4 (276)	-1.62	0.105
IA2b				
Male	180.5 (192)	219.0 (208)	-3.32	0.001
Female	69.6 (80)	80.3 (68)	-1.52	0.130
Combined	250.4 (272)	298.3 (276)	-3.54	0.0007
GAD				
Male	188.2 (192)	211.9 (208)	-2.05	0.04
Female	75.3 (80)	73.5 (68)	0.26	0.797
Combined	263.4 (272)	285.4 (276)	-1.63	0.104

* Mann-Whitney *U* test; p-value of <0.017 set for rejection of null hypothesis

Table 6. Association between antipsychotic medication and circulating IgG against IA2 and GAD antigens

Drug	Regression	IA2a	IA2b	GAD
Amisulpride	Adj r^2	-0.009	0.010	0.012
	t	0.269	0.062	0.861
	p	0.768	0.950	0.390
Clozapine	Adj r^2	-0.009	0.021	0.012
	t	0.280	-1.770	-0.900
	p	0.779	0.078	0.369
Haloperidol	Adj r^2	-0.009	0.012	0.013
	t	0.288	0.826	0.908
	p	0.773	0.409	0.365
Olanzapine	Adj r^2	-0.008	0.017	0.020
	t	0.707	1.436	1.657
	p	0.480	0.152	0.099
Phenothiazine	Adj r^2	-0.010	0.013	0.009
	t	0.039	0.926	-0.059
	p	0.969	0.355	0.953
Quetiapine	Adj r^2	-0.006	0.012	0.010
	t	-0.925	-0.734	-0.441
	p	0.356	0.464	0.659

Risperidone	Adj r^2	-0.010	0.010	0.016
	t	0.277	-0.507	-1.384
	p	0.782	0.613	0.168
Sulpiride	Adj r^2	0.010	0.011	0.023
	t	-0.087	0.550	1.893
	p	0.930	0.583	0.059
Thioxanthine	Adj r^2	-0.009	0.018	0.015
	t	0.393	1.518	1.215
	p	0.694	0.130	0.226

Adj r^2, adjusted r^2 for sex and age.

Table 7. Association between the DQ2.5 variant and plasma IgG against IA2 and GAD antigens

Subjects	**Antigen**	**Log-likelihood**	**G^2 statistic**	**p ***
Patient				
	IA2a	1.331	2.66	0.103
	IA2b	1.720	3.44	0.086
	GAD	1.722	3.44	0.086
Control				
	IA2a	0.197	0.39	0.530
	IA2b	0.011	0.02	0.885
	GAD	0.275	0.55	0.459

* The p-value of <0.017 was set for rejection of null hypothesis

Table 8. Association between the DQ8 variant and plasma IgG against IA2 and GAD antigens

Subjects	**Antigen**	**Log-likelihood**	**G^2 statistic**	**p ***
Patient				
	IA2a	2.217	4.43	0.035
	IA2b	0.095	0.19	0.663
	GAD	0.889	1.78	0.182
Control				
	IA2a	0.203	0.41	0.524
	IA2b	0.001	0.01	0.909
	GAD	0.0005	0.00	0.976

* The p-value of <0.017 was set for rejection of null hypothesis

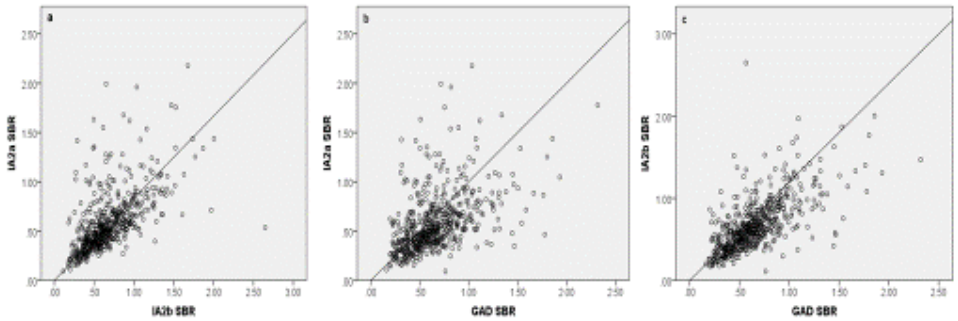

Figure 1. The correlation between circulating levels of IgG antibodies against linear peptide antigens derived from GAD and IA2

a. IA2a vs IA2b: r=0.591, df=547, p<0.001;
b. IA2a vs GAD: r=0.480, df=547, p<0.001;
c. IA2b vs GAD: r=0.663, df=547, p<0.001.