

CLINICAL STUDY

The role of functionally defective rare germline variants of sialic acid acetyltransferase in autoimmune Addison's disease

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Abstract

Background: Autoimmune Addison's disease (AAD) is a rare condition with a complex genetic basis. A panel of rare and functionally defective genetic variants in the sialic acid acetyltransferase (*SIAE*) gene has recently been implicated in several common autoimmune conditions. We performed a case–control study to determine whether these rare variants are associated with a rarer condition, AAD.

Method: We analysed nine *SIAE* gene variants (W48X, M89V, C196F, C226G, R230W, T312M, Y349C, F404S and R479C) in a United Kingdom cohort of 378 AAD subjects and 387 healthy controls. All samples were genotyped using Sequenom iPLEX chemistry to characterise primer extension products.

Results: A heterozygous rare allele at codon 312 (312*M) was found in one AAD patient (0.13%) but was not detected in the healthy controls. The commoner, functionally recessive variant at codon 89 (89*V) was found to be homozygous in two AAD patients but was only found in the heterozygous state in controls. Taking into account all nine alleles examined, 4/378 (1.06%) AAD patients and 1/387 (0.25%) healthy controls carried the defective *SIAE* alleles, with a calculated odds ratio of 4.13 (95% CI 0.44–97.45, two-tailed *P* value 0.212, NS).

Conclusion: We demonstrated the presence of 89*V homozygotes and the 312*M rare allele in the AAD cohort, but overall, our analysis does not support a role for rare variants in *SIAE* in the pathogenesis of AAD. However, the relatively small collection of AAD patients limits the power to exclude a small effect.

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Introduction

Autoimmune Addison's disease (AAD) is a rare autoimmune endocrinopathy, with a prevalence of one in 8000 people in the United Kingdom (1). It is commonly associated with autoimmune thyroid disease (~50%) and/or type 1 diabetes (~10%), constituting the type 2 polyendocrinopathy syndrome (2). In common with many other autoimmune conditions, AAD is believed to have a complex genetic basis, with the risk to first-degree relatives of about 2% and a $\lambda_s > 150$ (ratio of risk to a sibling vs the unrelated background population) (3). Candidate gene studies have identified numerous susceptibility alleles contributing to AAD, such as the MHC locus on chromosome 6p21, which harbours the strongest susceptibility variant(s) for this disorder, as well as loci *PTPN22* (4), *CTLA4* (5), *CLEC16A* (6), *CIITA* (6), *PDL1* (7), *CYP27B1* (8) and *NLRP1* (9, 10). Nevertheless, given the high genetic load of this rare condition, there are likely to be many more susceptibility alleles for AAD, which have yet to be elucidated. A knowledge of these variants is essential for improving our understanding of

AAD pathogenesis, with the ultimate aim of designing a small molecule or protein-targeted therapy as a translational medicine.

Recently, a panel of rare and functionally defective genetic variants in the sialic acid acetyltransferase (*SIAE*) gene were identified, in a high-profile publication, as being strongly associated with many autoimmune conditions, including Crohn's disease, type 1 diabetes, systemic lupus erythematosus (SLE), Sjogren's syndrome, juvenile idiopathic arthritis, multiple sclerosis (MS), mixed connective tissue disease, rheumatoid arthritis and ulcerative colitis (odds ratio (OR) > 8) (11). However, a subsequent larger study of more prevalent autoimmune and inflammatory disorders, including type 1 diabetes, coeliac disease, Crohn's disease and autoimmune thyroid disease, failed to replicate this finding (12). Nevertheless, *SIAE* represents one of the very first associations of rare genomic variants with common autoimmune disorders. Interestingly, loss-of-function rare variants in the *TREX1* (the major mammalian 3'–5' exonuclease) and *CYP27B1* (vitamin D 1 α -hydroxylase) genes have been described in SLE and MS patients respectively, suggesting that in

contrast to the common disease-common variant hypothesis, there may be a greater role for rare genetic variants in the susceptibility to less prevalent autoimmune diseases (13, 14).

SIAE is a negative regulator of B lymphocyte signalling by acting at inhibitory receptors that attenuate B-cell receptor signalling. Spontaneous auto-antibody production has been demonstrated in SIAE-mutant mice on a C57Bl/6 background, suggesting that defects in SIAE function conferred by SIAE variants might contribute to human autoimmunity (15). Genome-wide studies have not demonstrated any association or linkage with SNPs at the SIAE locus in patients with autoimmunity; however, under a multiple rare variants model, this might not be expected. Nevertheless, AAD is rarer than all the previously studied disorders and hence rare variants might have a significant contribution to its pathogenesis. The purpose of our study is to explore whether these rare SIAE variants are associated with AAD, which has not previously been studied (11, 12, 16).

Materials and methods

Three hundred and seventy-eight Caucasian subjects with AAD have been recruited since 1996 through outpatient endocrinology services in the North East of England and the UK Addison's disease self-help group. The diagnosis of AAD was confirmed by either a subnormal response to the ACTH₁₋₂₄ stimulation test (using 250 µg of parenteral synthetic ACTH), or a low basal cortisol with a high ACTH level. Patients with APS1, primary adrenal failure owing to infiltrative or infective causes or secondary adrenal failure were excluded. Three hundred and eighty-seven healthy local Caucasian controls were used for comparison (including 113 individuals from a 1958 birth cohort). This study was carried out with approval of the Leeds (East) Research Ethics Committee (Ref 05/Q1206/144).

Methods

We studied nine rare germline variants within the SIAE gene (chromosome 11), which are among the 12 rare non-synonymous SIAE variants demonstrated by Surolija *et al.* to be functionally defective in esterase activity or enzyme secretion (11). These rare SNPs comprise 21 of the 24 cases (88%) of functionally defective rare SIAE variants reported by Surolija *et al.* The SNPs selected include C196F, T312M, C226F, F404S, R230W, R479C, W48X, Y349C and M89V. These SNPs were selected and genotyped in Caucasian individuals with AAD and healthy controls in the UK.

Genomic DNA was extracted from venous blood from each subject and used for multiplex PCR at a concentration of 20 ng/µl. PCR was performed in a 10 µl reaction volume using a Qiagen PCR kit, using the following concentrations per reaction: 20 ng/µl genomic DNA, 1.25× PCR buffer, 25 mM magnesium chloride, 0.5 µM of each primer, 6.25 mM of each dNTP, and 5 U/µl HotStar Taq DNA polymerase. Primer sequences are available from the authors on request. PCR was then performed by initial denaturation for 15 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 3 min. Amplified products were then genotyped using Sequenom iPLEX chemistry for MALDI-TOF characterisation of primer extension products. Ten to 15% of all samples were re-genotyped blind for each assay to ensure fidelity of genotyping (>99% for each SNP). The results were verified by direct DNA sequencing where appropriate, using Eurofins MWG Operon's DNA sequencing service.

Statistical analysis and power

Fisher's exact test was used for association analysis, by means of 2×2 contingency tables. Control genotypes were checked for Hardy-Weinberg Equilibrium (threshold $P>0.05$). A power estimation, using the

Table 1 Genotypes for SIAE variants in autoimmune disease cases and controls.

	Autoimmune Addison's disease		UK healthy controls		P value
	Genotype ^a	Rare allele/genotype frequency	Genotype ^a	Rare allele frequency	
W48X	0/0/370	0	0/0/373	0	–
M89V	2/42/303	0.0058 ^b	0/43/315	0	0.242
C196F	0/1/374	0.0013	0/1/383	0.0013	–
C226G	0/0/371	0	0/0/383	0	–
R230W	0/0/375	0	0/0/386	0	–
T312M	0/1/372	0.0013	0/0/385	0	0.492
Y349C	0/0/360	0	0/0/381	0	–
F404S	0/0/372	0	0/0/382	0	–
R479C	0/0/358	0	0/0/362	0	–

^aData presented as number of rare homozygote/heterozygote/common homozygote genotypes.

^bHomozygous 89°V genotype.

Table 2 Clinical details of AAD patients with rare SIAE variants.

SIAE variants	Age at onset of AAD (years)	Associated autoimmune conditions
M89V (homozygous)	29	Pernicious anaemia
M89V (homozygous)	60	Pernicious anaemia, autoimmune hypothyroidism, premature ovarian failure
C196F	83	Autoimmune hypothyroidism
T312M	59	Pernicious anaemia

pooled case and control allele frequencies found by Surolija *et al.* (2.6 and 0.3% respectively) (11), showed that our study design had 78% power to detect a similar-sized effect ($\alpha=0.05$).

Results

Among the 761 subjects (378 cases and 387 controls), we did not find any rare allele variants for the SIAE germline variants encoding W48X, C226G, R230W, Y349C, F404S and R479C (Table 1). One AAD patient was a heterozygous carrier of the codon 312*M (T312M) variant, but all controls were found to have the wild-type allele. A single heterozygous carrier of the SIAE 196*F (C196F) allele was found amongst both the case and the control cohorts. As previously found (11), the codon 89*V allele was present heterozygously in 12.1 and 12.0% of the patient and control cohorts respectively. However, no codon 89*V homozygotes were found amongst control patients, whereas two AAD patients were 89*V homozygotes ($P=0.242$).

All AAD patients who are either heterozygous carriers of the rare codon 312*M (T312M) and 196*F (C196F) variants, or the homozygous carriers of the codon 89*V (M89V), have other associated autoimmune diseases in the spectrum of type 2 autoimmune polyendocrinopathy syndrome (APS2). Among them, three had pernicious anaemia, two autoimmune hypothyroidism and one premature ovarian failure (Table 2).

In summary, taking into account all nine alleles examined, 4/378 (1.06%) AAD patients and 1/387 (0.25%) healthy controls inherited SIAE genotypes that would be expected to lead to functionally detrimental consequences; OR of 4.13 (95% CI 0.44–97.45; two-tailed P value 0.212, NS).

Discussion

The association between rare SIAE genetic variants and disease susceptibility has recently been explored in some common autoimmune diseases, with conflicting results

among various research groups (11, 12, 16, 17). Nevertheless, the loss-of-function rare variants in the *TREX1* and *CYP27B1* genes have been significantly implicated in SLE and MS patients respectively (13, 14), suggesting the possibility that rare genetic variants may have a role in risk susceptibility for some of the less prevalent autoimmune diseases. We explored the hypothesis that rare SIAE variants would be associated with AAD, one of the least common autoimmune conditions. We demonstrated the presence of two codon 89*V homozygotes and one heterozygous carrier of the 312*M allele in the AAD cohort, which was not significantly different from healthy control genotypes. Thus, our findings extend the negative study of Hunt *et al.* (12) who showed no differences for nine of the SIAE gene rare variants (including M89V but not T312M) in cohorts of the commoner autoimmune and inflammatory conditions, such as type 1 diabetes, atopic eczema, celiac disease, Graves' disease and Hashimoto thyroiditis. However, this large cohort of nearly 67 000 subjects did not include patients with the rarer condition of AAD.

While our study had good power to replicate findings of a similar magnitude to those previously seen in other autoimmune conditions (OR 8.6) (11), our analysis is underpowered to detect a more subtle genetic effect, such as is more frequently seen for commoner autoimmune disease susceptibility alleles. We cannot exclude that a future analysis of an enlarged AAD cohort or a family-based genetic association study may cast further light on this question. However, the parameters for declaring significance in studies of rare genetic variants are not yet well defined (18), and the key attribute of reproducibility has not been fulfilled for SIAE variants in autoimmune diseases to date.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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