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Short Communication

Tri-SNP polymorphism in the intron of HLA-DRA1 affects type 1 diabetes susceptibility in the Finnish population

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ABSTRACT

Genes in the HLA class II region include the most important inherited risk factors for type 1 diabetes (T1D) although also polymorphisms outside the HLA region modulate the predisposition to T1D. This study set out to confirm a recent observation in which a novel expression quantitative trait locus was formed by three single nucleotide polymorphisms (SNP) in the intron of HLA-DRA1 in DR3-DQ2 haplotypes. The SNPs significantly increased the risk for T1D in DR3-DQ2 homozygous individuals and we intended to further explore this association, in the Finnish population, by comparing two DR3-DQ2 positive genotypes. Cohorts with DR3-DQ2/DR3-DQ2 (N = 570) and DR3-DQ2/DR1-DQ5 (N = 1035) genotypes were studied using TaqMan analysis that typed for rs3135394, rs9268645 and rs3129877. The tri-SNP haplotype was significantly more common in cases than controls in the DR3-DQ2/DR3-DQ2 cohort (OR = 1.70 CI 95% = 1.15–2.51P = 0.007). However, no significant associations could be observed in the DR3-DQ2/DR1-DQ5 cohort.

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1. Introduction

Type 1 diabetes (T1D) has a multifactorial etiology attributable to genetic and environmental factors that affect the development of islet autoimmunity and the progression of β-cell destruction. The strongest observable T1D-susceptibility factors are polymorphisms in HLA class II genes, especially DRB1, DQA1 and DQB1 but also DPB1 [1,2]. Moreover, genetic variants of HLA class I alleles, including A*24, B*18, and B*39 are also associated with T1D [3]. Still, numerous poorly characterized polymorphisms in the HLA-region have been found to modify T1D susceptibility [4]. >40 single nucleotide polymorphisms (SNP) outside the HLA-region have been associated with T1D. However, these SNPs have only a minor effect on the disease risk, when compared to the strong effect of HLA-DR/DQ susceptibility factors [5,6].

A recent study by Aydemir et al. described three novel SNP in intron-1 of HLA-DRA1 which modulated the T1D-risk conferred by DRB1*03-DQA1*05-DQB1*02 haplotypes in a mixed study group consisting of European ancestry study subjects from the Type 1 Diabetes Genetic Consortium (T1DG) and from a Swedish cohort [7]. They suggested that the combined "tri-SNP" haplotype functions as an expression quantitative trait locus (eQTL) significantly increasing T1D susceptibility. The tri-SNP combination consisting of three risk alleles (rs3135394-A, rs9268645-G, and rs3129877-G) was significantly associated with T1D in both tri-SNP heterozygotes and homozygotes.

This study aimed to further investigate the T1D-risk conferred by the tri-SNP in a large Finnish cohort. In addition to studying (DR3)-DQA1*05-DQB1*02 (DR3-DQ2) homozygotes, the most com-

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mon heterozygous DR3-DQ2 genotype, DR3-DQ2/(DR1/10)-DQB1*05:01 (DR1-DQ5) was selected for further study [8].

According to a DR/DQ risk estimation of trio families in the Finnish Pediatric Diabetes Register (FPDR) is the DR3-DQ2/DR3-DQ2 genotype conferring a moderately increased risk (OR = 4.08 CI 95% = 2.49–6.69) while the DR3-DQ2/DR1-DQ5 genotype confers only a slightly increased risk (OR = 1.42 CI 95% = 1.09–1.85) for T1D [8]. We hypothesized that the tri-SNP could be used as an additional risk factor when estimating T1D susceptibility in prospective genetic screening.

2. Materials and methods

2.1. Study population and cohorts

Samples from children with T1D were obtained from the FPDR and the healthy controls were obtained from children screened for eligibility in the Finnish Diabetes Prediction and Prevention study (DIPP). Most pediatric units in Finland are participating in the FPDR, and the sample library is perceived to represent the complete population diversity of individuals with T1D [9]. The DIPP-study recruits newborn infants for HLA-based genetic screening in three university hospitals (Turku, Oulu, and Tampere) in Southwest, Middle and Northern Finland, screening approximately 25% of all births annually [8].

In the DR3-DQ2/DR3-DQ2 cohort there were 190 T1D DNA samples obtained from the FPDR and 380 control samples from the DIPP study. The controls had not developed islet specific autoantibodies (IAA, GADA or IA-2A) or T1D during the follow-up. The DR3-DQ2/DR1-DQ5 cohort included 384 DNA samples from children diagnosed with T1D in the FPDR and 651 DIPP blood spot samples from subjects who were screened for DQB1 but who were not eligible for follow-up study. None of the controls have been diagnosed with T1D in the participating hospitals by the end of year 2017. The male to female sex ratio for the DR3-DQ2/DR3-DQ2 cohort was 64.7% and 35.3% in cases and 53.2% and 46.8% in controls, respectively. Corresponding ratios in the DR3-DQ2/DR1-DQ5 cohort was 51.7% and 48.3% in cases and 50.8% and 49.2% in controls. This study was authorized by the ethics committees of the participating hospitals in accordance with the Declaration of Helsinki. All samples and data were collected with informed consent from the legal guardians and stored according to the protocols of the DIPP-study and the FPDR.

2.2. HLA genotyping

HLA class II genotyping was tailored to define major haplotypes associated with a risk for or protection against T1D. Typing was initiated with full-house typing for major DQB1 alleles, with four-digit resolution, followed by a panel of probes defining three informative DQA1 alleles (DQA1*02:01, DQA1*03, DQA1*05) discriminating between major haplotypes containing DQB1*02, DQB1*03:01 and DQB1*03:03 alleles. DRB1*04 alleles associated with variable T1D-risk were further defined in DQB1*03:02 positive samples. The haplotypes are presented by their abbreviated names, complemented by DR specificities (in parenthesis), which were deduced from determined linkages in populations of European descent [10]. A set of DQB1 genotypes had their second exon hypervariable region sequenced so that alternative genotypes could be distinguished. The DQB1-assay is an in-house homogeneous assay using asymmetric PCR wherein the DQB1 gene is amplified with lanthanide labelled probes and complementary quencher oligonucleotides. Probes and quenchers hybridize after amplification by cooling in room temperature followed by lanthanide measurement by time-resolved fluorometry [11].

The HLA-DRB1 assays and a part of the DQA1 assays were performed using the DELFIA method [11,12]. DQA1 typing in recent samples, including the blood spot samples with DQB1*02/DQB1*05:01 genotypes, was performed using a homogeneous end-point PCR assay with fluorescence resonance energy transfer (FRET). The method is based on two non-luminescent oligonucleotide probes, defining each allele: one carrying a non-luminescent lanthanide chelate and the other carrying a light-absorbing antenna ligand. Hybridization of probes to adjacent positions on target DNA leads to the formation of a highly luminescent lanthanide chelate complex by reporter molecule self-assembly [13]. Four digit typing for DQA1*05 and DQB1*02 alleles was not performed since the haplotype formed by them in populations with European ancestry invariably consists of DQA1*05:01 and DQB1*02:01 alleles whereas other common DQA1*05 alleles DQA1*05:05 and DQA1*05:03 are found in haplotype with DQB1*03:01 [14].

2.3. Tri-SNP genotyping

DNA extraction from EDTA-blood was performed according to a standard salting-out protocol [15]. DNA extraction from bloodspot-cards was done by extraction and solution preparation according to standard in-house protocol [11]. Undiluted stock samples and samples extracted from EDTA blood with visible impurities were purified with a Nucleospin™ gDNA Clean-up kit (Macherey-Nagel, ref. 740230.50) according to the manufacturers protocol. All DNA concentrations were measured with a Qubit™ 4 Fluorometer (Thermo Fisher, cat. Q33226). The DR3-DQ2/DR1-DQ5 cohort required additional HLA-typing due to lacking DQA1-data. DQA1-typing was performed with homogenous asymmetric PCR using lanthanide labelled oligonucleotide probes according to standard in-house protocol [12]. Only DQA1*05-positive subjects were included in the final DR3-DQ2/DR1-DQ5 cohort.

SNP genotyping was performed by TaqMan qPCR using on-the-shelf TaqMan™ probes (Thermo Fisher, cat. 4351379) for rs3135394 (Assay ID: C__27466166_20), rs9268645 (Assay ID: C__27466166_20) and rs3129877 (Assay ID: C__27466166_20). The contents of the reaction solution were modified from the manufacturers protocol to 2.5 μl TaqMan™ Genotyping Master Mix (Thermo Fisher, cat. 4371355), 0.06 μl of either 40x TaqMan™ probe, 1.44 μl MQ-water and 1 μl DNA (approx. 10 ng/μl). The qPCR-reaction utilized a 96-well semi-skirted Framestar® PCR-plate (4titude, prod. 4ti-0770/C) with Framestar® qPCR seals (4titude, prod. 4ti-0560). The qPCR was run on a QuantStudio™ 3 Real-Time PCR system (Thermo Fisher, cat. A28317) with a 96-well 0.2 ml block. qPCR-amplification used preprogrammed settings adjusted to a 10 μl reaction volume and to run for 50 cycles. Reruns of undetermined samples used a 25% enlarged reaction volume with 2 μl DNA. Reruns of impure samples with observable impurities used only 1 μl DNA. The genotyping results were analyzed with QuantStudio™ Designs & Analysis Desktop Software (v1.5.1) with analytical call settings set at *analyze Real-time Rn-Median (Rna to Rnb)*. A summary of all calls intended for analysis was made with Thermo Fishers Connect™ cloud software for genotyping with previously specified call settings.

2.4. Statistics

SNP frequencies were compared and P-values calculated using Pearson Chi-square or Fishefs exact test when applicable. Linkage disequilibrium values between respective tri-SNP components were calculated using PLINK (v1.07) program [16] in DR3-DQ2 homozygote cohorts and using LDlink [17] in the Finnish excerpt of 1000Genomes.

3. Results

Table 1 shows the frequency distribution of tri-SNP genotypes in the DR3-DQ2/DR3-DQ2 cohort. GCA was the dominant haplotype and GCA homozygosity was the most common genotype with a frequency of 75.8% in cases and 81.1% in controls. Homozygosity for the T1D-associated AGG genotype was rare, although twice as common in cases (5.3%) than controls (2.6%), albeit not significantly different. The second most frequent genotype was AG-CG-AG, an apparent combination of the AGG and GCA haplotypes, which was more common in cases than in controls (P = 0.066). Other genotypes were rare, only 4/190 (2.1%) in cases and 19/380 (5.0%) in controls had a combination different from GCA or AGG. In the rare genotypes the AGG combination was present as a probable genotype in one case and three controls whereas the GCA combination was found in two cases and 12 controls (Table 1). When the frequency of all cases with a possible AGG-genotype 43/190 (22.6%) was compared to the controls 56/380 (14.8%) could a significant difference be detected (OR = 1.69 CI 95%= 1.09–2.63P = 0.019) (Table1).

When the three most common tri-SNP genotypes were analyzed independently, as done by Aydemir et al. [7], a trend with considerable overlapping values could be observed (Table 1). The frequency of children with a single or a double AGG haplotype was significantly more common in cases (OR = 1.70 CI 95%= 1.15–2.51, P = 0.007) than in the controls. An analysis of the SNPs allelic frequency distributions in the DR3-DQ2/DR3-DQ2 cohort showed a significant increase in minor rs9268645-G (OR = 1.65 CI 95%= 1.13–2.42P = 0.010) and minor rs3129877-G (OR = 1.48 CI 95%= 1.03–2.13P = 0.034) amongst the cases (data not shown). The minor rs3135394-A was more common in cases but showed only a borderline significance (OR = 1.43 CI 95%= 0.99–2.06P = 0. 054).

No AGG homozygotes were present in the DR3-DQ2/DR1-DQ5 cohort leading to a strong dominance of GCA haplotypes (Table 2). However, the AG-CG-AG genotype (probable AGG/GCA) was slightly more frequent in cases than in controls, albeit the difference was not significant. By comparing all cases with a possible AGG-genotype 49/462 (10.6%) against the controls 48/573 (8.4%) no significant difference could be detected (OR = 1.30 CI 95%= 0.8 5–1.97P = 0.221). Similarly, no significant differences in allelic distribution could be observed in the DR3-DQ2/DR1-DQ5 cohort (data not shown).

Linkage disequilibrium (LD) values for respective SNPs were analyzed in DR3-DQ2 homozygote cases and controls as well as in the Finnish general population data retrieved from the 1000Genomes collection [18]. LD-analysis of DR3-DQ2 SNP data using PLINK (v1.07) found a strong LD between all SNPs (D'> 0.9) and allele frequencies were in strong correlation ($r^2 > 0.8$), demonstrating that the tri-SNP components are inherited together and seldomly recombined (Table 3). A strong LD between all SNPs (D'=1) was also found in the 1000Genomes dataset but the allele frequencies were noticeably disproportionate and only a weak correlation was found between them ($r^2 = 0.02–0.22$), indicating that the tri-SNP components are inherited from several different haplotypes (Table 4). This is also demonstrated by the fact that the minor allele frequencies of respective SNP (rs3135394, rs9268645, rs3129877) in the DR3-DQ2 cohort (0.11, 0.089, 0.11) clearly deviate from the Finnish general populations minor allele frequencies (0.084, 0.29, 0.41), according to data obtained from the Genome Aggregation Database (gnomAD) [19].

4. Discussion

This study verified that the tri-SNP in the intron of HLA-DR3 gene of DR3-DQ2 haplotype has a significant effect on the risk for T1D development as first reported in 2019 [7]. The association was detectable in Finnish DR3-DQ2/DR3-DQ2 homozygotes, where the AGG-haplotype conferred an increased risk for T1D. The association followed a similar pattern to the original study, in which

Table 1
Summary of genotypes and haplotypes in the DR3-DQ2/DR3-DQ2 cohort. Genotype combinations are arranged in the order of minor to major SNP, rs3135394, rs9268645 and rs3129877 respectively. Genotypes compare combinations resulting in a determined A-G-G genotype in comparison to other genotypes without a complete A-G-G combination. Haplotypic odds ratios and p-values are relative to the lower risk GCA/GCA haplotype. Odds ratios and P-values were calculated using Pearson Chi-square, P-values with Fisher's exact test when applicable.

DR3-DQ2/DR3-DQ2				
Genotypes combinations	Cases (%)	Controls (%)	OR (95% CI)	P-value
AA-GG-GG	10 (5.3)	10 (2.6)	2.06 (0.84–5.03)	0.108
AA-CG-GG	1 (0.5)	1 (0.3)		
AA-CG-AG		2 (0.5)		
AA-CC-AG		1 (0.3)		
AG-CG-AG	32 (16.8)	43 (11.3)	1.59 (0.97–2.60)	0.066
AG-CG-AA		1 (0.3)		
AG-CC-AG	1 (0.5)	6 (1.6)		
AG-CC-AA	1 (0.5)	4 (1.1)		
GG-CG-AG		1 (0.3)		
GG-CC-GG	1 (0.5)	3 (0.8)		
GG-CC-AA	144 (75.8)	308 (81.1)	0.73 (0.48–1.11)	0.144
Genotypes A-G-G	43 (22.6)	56 (14.8)	1.69 (1.09–2.63)	**0.019**
Other	147 (77.4)	324 (85.7)	0.59 (0.38–0.92)	**0.019**
Total N	190	380		
Haplotype combinations	Cases (%)	Controls (%)	OR (95% CI)	P-value
AGG/AGG	10 (5.4)	10 (2.8)	2.14 (0.87–5.25)	0.090
GCA/AGG*	32 (17.2)	43 (11.9)	1.59 (0.97–2.62)	0.066
GCA/GCA	144 (77.4)	308 (85.3)	1.00 (NA)	NA
Total N	186	361		
Haplotypes AGG	52 (14.0)	63 (8.7)	1.70 (1.15–2.51)	**0.007**
GCA	320 (86.0)	659 (91.3)	1.00 (NA)	NA
Total N	372	722		

*Deduced haplotypes in AG-CG-AG genotypes.

Table 2
Summary of genotypes and haplotypes in the DR3-DQ2/DR1-DQ5 cohort. Genotype combinations are arranged in the order of minor to major SNP, rs3135394, rs9268645 and rs3129877 respectively. Genotypes compare combinations resulting in a determined A-G-G genotype in comparison to other genotypes without a complete A-G-G combination. Haplotypic odds ratios and p-values are relative to the lower risk GCA/GCA haplotype. Odds ratios and P-values were calculated using Pearson Chi-square, P-values with Fisher's exact test when applicable.

DR3-DQ2/DR1-DQ5				
Genotype combinations	Cases (%)	Controls (%)	OR (95% CI)	P-value
AG-GG-GG	1 (0.2)			
AG-CG-GG	1 (0.2)	1 (0.2)		
AG-CG-AG	47 (10.2)	47 (8.2)	1.27 (0.83–1.94)	0.273
AG-CC-GG		1 (0.2)		
AG-CC-AG		3 (0.5)		
GG-GG-AA		1 (0.2)		
GG-CG-GG	1 (0.2)			
GG-CG-AG	4 (0.9)	1 (0.2)		
GG-CC-AG	4 (0.9)	11 (1.9)		
GG-CC-AA	404 (87.4)	508 (88.7)	0.89 (0.61–1.30)	0.550
Genotypes A-G-G	49 (10.6)	48 (8.4)	1.30 (0.95–1.97)	0.221
Other	413 (77.2)	525 (91.6)	0.77 (0.51–1.17)	0.221
Total N	462	573		
Haplotype combinations	Cases (%)	Controls (%)	OR (95% CI)	P-value
AGG/AGG				
GCA/AGG*	47 (10.4)	47 (8.5)	1.26 (0.82–1.92)	0.291
GCA/GCA	404 (89.6)	508 (91.5)	1.00 (NA)	NA
Total N	451	555		
Haplotypes AGG	47 (5.2)	47 (4.2)	1.24 (0.82–1.88)	0.302
GCA	855 (94.8)	1063 (95.8)	1.00 (NA)	NA
Total N	902	1110		

*Deduced haplotypes in AG-CG-AG genotypes.

Table 3
Linkage disequilibrium between analyzed SNPs in the DR3-DQ2/DR3-DQ2. Respective case and control dataset LD-value is presented in the case/control field. The whole-cohort LD-value was calculated with frequency data from both the case and the control dataset. The summarized LD-result is presented below the case/control value. LD-values were calculated with PLINK.

DR3-DQ2/DR3-DQ2			
r^2	rs3135394	rs9268645	rs3129877
rs3135394	1/1	0.938/0.786	0.939/0.803
rs9268645	0.938/0.786	1/1	0.918/0.796
rs3129877	0.939/0.803	0.918/0.796	1/1
D′	rs3135394	rs9268645	rs3129877
rs3135394	1/1	1/0.983	0.979/0.902
rs9268645	1/0.983	1/1	1/0.983
rs3129877	0.939/0.803	0.918/0.796	1/1

Table 4
Linkage disequilibrium between tri-SNPs alleles in a 1000Genomes excerpt. Values were retrieved from the 1000Genomes Finnish population data collection using LDlink.

1000Genomes			
r^2	rs3135394	rs9268645	rs3129877
rs3135394	1	0.023	0.103
rs9268645	0.023	1	0.225
rs3129877	0.103	0.225	1
D′	rs3135394	rs9268645	rs3129877
rs3135394	1	1	1
rs9268645	1	1	1
rs3129877	1	1	1

the AGG-heterozygote formed an intermediate risk group and the AGG-homozygote a higher risk group. No significant associations were found in the DR3-DQ2/DR1-DQ5 cohort. However, the insignificant results of reported allele, genotype, and haplotype frequencies resulted in odds ratios with a direction similar to those in the DR3-DQ2/DR3-DQ2 cohort. Moreover, LD-analysis of the three SNPs presented a strong LD (D'> 9), which was to be expected since the tri-SNP components are residing within a 100 bp region. However, the strong difference in correlation coefficients values, when comparing r^2-values provided by the 1000Genomes dataset (r^2 = 0.02–0.22) with the DR3-DQ2 dataset (r^2 > 0.8), would suggest that the SNPs are in a moderate LD with the DR3-DQ2 haplotype.

A limitation of this study is that all tri-SNP haplotypes cannot be deductively determined based on our method of SNP-typing. This limitation includes AG-CG-AG (AGG/GCA), which was the second most common genotype in the study of Aydemir et al. [7]. The DR3-DQ2 relation to AGG/GCA in the DR3-DQ2/DR1-DQ5 cohort cannot be determined without sequencing. However, the dominance of GCA homozygotes in both cohorts and the rare presence of AGG combinations in other genotypes, besides AG-CG-AG, deductively suggest that the AG-CG-AG genotype can be interpreted as the heterozygous AGG/GCA genotype.

Based on a 1000 genome data analysis Aydemir and coworkers suggested that the tri-SNP affects HLA-DQB1 allele expression as an eQTL, a function which might explain the increased T1D-risk [7]. Specific alleles associated with T1D in both DRB1 and DQB1 loci have been found to encode molecules able to present islet antigen derived peptides to T lymphocytes [20] and an incrased expression detected of these HLA molecules in antigen presenting cells [21] could also enhance this antigen presentation.

However, neither that report nor this study have considered allele specificity in other loci within the studied haplotypes, like HLA-DP, class I HLA loci or other HLA-polymorphisms. The DR3-DQ2 haplotype conferred risk has been found to be dependent on factors outside the DR/DQ loci including the HLA-DP loci [2]. Furthermore, HLA-B18 positive DR3-DQ2 haplotypes have been observed to be more susceptible to T1D than B8 positive haplotypes [22]. Several other polymorphisms as SNPs in DMB and DOB genes, and microsatellite locus TNFc have also been associated with T1D; demonstrating risk effects which are independent of DRB1, DQA1 and DQB1 [23].

It should be noted that the frequency of risk-associated tri-SNP haplotypes in our population of DR3-DQ2 homozygous children with T1D (14%) was clearly lower than the 30.9% described amongst Swedish cases in the earlier study [7]. When compared to many other European populations is the HLA-diversity in Finland quite low. This is an issue when studying T1D-related risk factors that could be restricted to rare HLA-haplotypes and are accordingly hard to identify in large numbers. For example, the DR3-DQ2 haplotype usually carries the lower risk B8 allele and very rarely the high-risk associated B18 allele [24,25]. The low frequency of high-risk associated DR3-DQ2 haplotypes, carrying the DR3-DQ2 restricted tri-SNP, reduce the practicability of tri-SNP inclusion in future risk screening programs as the addition would not significantly enhance the sensitivity and the specificity of the genetic screening.

By eliminating HLA-heterogeneity we were able to detect the tri-SNPs risk effect in a DR3-DQ2/DR3-DQ2 cohort, but not in the DR3-DQ2/DR1-DQ5 cohort. The tri-SNP haplotype could be deductively determined in homozygous genotypes eliminating the need for sequencing. However, HLA heterozygosity and the low frequency of respective tri-SNPs minor allele in the DR3-DQ2/DR1-DQ5 cohort reduced the applicability of the results. To conclude, we demonstrated that the earlier reported tri-SNP haplotype, in the intron of HLA-DRA1 of DR3-DQ2 haplotype, is significantly associated with T1D risk in Finnish DR3-DQ2 homozygotes.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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