Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations

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Abstract. We have developed high-throughput tests for the detection of the insulin gene region SNPs -23HphI and -2221MspI. The potential of these markers to enhance the efficiency of type 1 diabetes risk screening was then evaluated by analyzing them in Finnish and Swedish populations. Blood spots on filter paper were analyzed using PCR followed by sequence-specific hybridization and time-resolved fluorometry reading. Distribution of the genotypes at both positions differed significantly among the affected children compared to the controls. The risk genotypes (CC, AA) were significantly more common in Finland than in Sweden, both among patients and controls. The VNTR genotype homozygous for the protective class III alleles showed a significantly stronger protective effect than the heterozygote ($p = 0.02$). Analyzing both SNPs enabled the detection of VNTR class III subclasses IIIA and IIIB. The observed significance between effects of the protective genotypes was due to the strong protective effect of the IIIA/IIIA genotype. IIIA/IIIA was the only genotype with significant discrepancy between protective effects compared to the other class III genotypes. These observations suggest that heterogeneity between the protective IDDM2 lineages could exist, and analyzing both -23HphI and -2221MspI would thus potentially enhance the sensitivity and specificity of type 1 diabetes risk estimation.

Keywords: Type 1 diabetes, insulin gene region, Finnish population, screening for genetic risk

1. Introduction

Susceptibility to type 1 diabetes (T1D) is defined largely by genetic constitution. The major role of the HLA genes constituting approximately half of the

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genetic component has been known since the 1970s, whereas evidence for additional gene regions with a smaller contribution has only slowly accumulated. Among these "minor" regions is the insulin gene region (IDDM2). The contribution of the insulin gene region to T1D susceptibility has been widely studied and the effect of the polymorphisms upon disease risk within the region is well established, although their exact function in the disease aetiology is still to be unravelled. IDDM2 locus has been mapped to a VNTR (variable number of tandem repeat minisatellite) locus located 596 bp upstream of the insulin gene (INS) translation starting codon [1]. SNPs adjacent to VNTR were excluded as aetiological polymorphisms [1], but a recent association study of 177 polymorphisms in and around the IDDM2 region reinstated two SNPs, −23HphI and +1140A/C, as potential candidates for causal variant [2].

VNTR consists of repeat units of 14–15 base pairs [3]. Class I VNTR alleles contain 28 to 44 repeats and class III alleles 138 to 159 repeats. Intermediate class II alleles are rare in non-African populations [4, 5].

As a group, class I alleles are associated with risk for T1D while class III alleles show a dominant protective effect, but both classes have individual alleles or subclasses with reportedly converse or neutral effects upon T1D risk [6,7]. Class I alleles are associated with significantly higher levels of insulin expression in the pancreas when compared to class III alleles [8,9]. Conversely, in thymus, the class III containing genotypes are associated with significantly higher levels of insulin expression (2 to 3 fold) than the class I homozygote. VNTR class-specific differences in thymic insulin expression is hypothesized to be the functional basis by which the IDDM2 locus affects T1D aetiology. Higher level of thymic insulin is assumed to lead to a better tolerance of insulin autoantigen and thus protection against T1D [10–12]. The mechanism by which VNTR allegedly regulates the levels of thymic insulin expression is unknown and the possibility that VNTR is simply a marker to adjacent functional polymorphism(s) has not been undeniably excluded.

SNPs −23HphI and +1140A/C are effectively in complete linkage disequilibrium (LD) with VNTR classes I and III [5], which allows them to be used as surrogate markers for VNTR classes. It is preferable to analyze the surrogate marker instead of the VNTR since genotyping the GC-rich VNTR minisatellite is a time-consuming technical challenge compared to the relatively mundane methods required to genotype the flanking SNPs.

Haplotypes of the VNTR class III alleles and neighbouring variations have been divided into two protective lineages, the protective haplotype (PH) and the very protective haplotype (VPH) [1], which correspond to VNTR class III subclasses IIIA and IIIB respectively [6]. The protective effect of PH and VPH have been shown to differ significantly in earlier studies [1–14], but a recent study by Barratt et al., using several independent populations, was unable to find a significant discrepancy between the protective effects conferred by these haplotypes [2].

Haplotypes of the flanking SNPs can be used to make a distinction between IIIA and IIIB without analyzing VNTR directly. Genotyping −2221MspI in addition to −23HphI allows the separation of the two subclasses with effective accuracy and reasonable ease [2–5]. Allele A of surrogate SNP −23HphI denotes VNTR class I, and allele T class III. Allele C of −2221MspI represents VNTR class I or subclass IIIB, while the T allele represents subclass IIIA (in 98% of the lineages in the UK population) [5]. Consequently, VNTR class I is defined by SNPs −2221MspI and −23HphI forming respective haplotype C-A, subclass IIIB (VPH) by haplotype C-T and subclass IIIA (PH) by haplotype T-T.

We have previously demonstrated the apparent effect of the INS gene −2221MspI polymorphism on the T1D risk in an extensive population-based series in Finland [15]. Since the VNTR class I associated −2221MspI C allele is also present in the protective class III haplotype, analyzing VNTR surrogate marker −23HphI instead of −2221MspI could enhance the sensitivity and specificity of IDDM2 in the T1D risk estimation. Also, if disparity in the levels of the protective effect from class III subclasses IIIA and IIIB exists, analyzing both markers would enable subclass separation and further enhance the accuracy of the T1D risk estimation.

For the purpose of evaluating the relative value of these markers in screening for the T1D risk, we have developed a high-throughput test for the detection of the −23HphI polymorphism and analyzed these two insulin gene SNPs in the Finnish and Swedish populations.

2. Subjects, materials and methods

Samples were collected at paediatric centres treating children affected by T1D. All subjects with T1D were diagnosed under the age of 15 according to the

WHO criteria. The study was approved by the Ethics Committees of the participating hospitals and informed consents were obtained from the study subjects and/or their parents. In 612 Finnish T1D families the affected child and both parents were genotyped. In each family, the parental haplotypes not transmitted to the affected child were used as affected family-based artificial controls (AFBAC) [16] and the transmitted haplotypes were considered as cases. The families were tested for inconsistencies between the parents and offspring with the PEDCHECK [17] program. Families showing possible non-paternity were removed from the AFBAC analysis.

The Finnish families with T1D originated from all around Finland. The Swedish cohorts were collected in Malmö (Southern Sweden): 142 children with T1D and 219 controls, and in Linköping (Southeastern Sweden): 77 children with T1D and 540 controls. An additional 138 children with T1D were sampled in Stockholm (Southeastern Sweden). All datasets were in Hardy-Weinberg equilibrium for both of the markers. The -2221MspI – -23HphI haplotypes were used to deduce the VNTR genotypes for each sample. Since parental data was not available for the Swedish cohorts, SNP haplotypes were constructed from the combined -23Hphi – -2221MspI genotypes. The haplotypes of combined genotypes were non-decipherable only in the combined heterozygote CT-AT. Fortunately, the haplotype which combines allele T at -2221MspI and allele A at -23HphI is extremely rare, so effectively the haplotypes for the CT-AT genotype were C-A and T-T.

All the Finnish samples and the Swedish samples from Linköping and Stockholm were analyzed using a sequence-specific oligonucleotide hybridization method based on lanthanide labeled probes. The Swedish samples from Malmö were analyzed with a hybridization method for the -2221MspI and with a HphI digestion method [18] for the -23HphI. There were no significant or conspicous differences between allele frequencies in the Swedish datasets analyzed by the different genotyping methods. The assay used in the analysis of the -2221MspI polymorphism has been described in detail earlier [15]. For the -23HphI assay, a circle of the blood spot was first incubated with sodium hydroxide and then neutralized with a Tris-Cl buffer. 20 μl of extract containing genomic DNA was transferred into the amplification mixture. The biotinylated amplification product was hybridized with two oligonucleotide probes after denaturation (T probe: 5'-Europium-CTGTCTCCCAGA-3' and A probe: 5'-Terbium-CTGTCACCCAGA-3'). Primers

used for the PCR amplification were: forward 5'-CTGGGCTCGTGAAGCATGT-3' and 5'-biotinylated reverse 5'-GCATCCACAGGGCCATG-3'. The consistency of the genotyping method was validated by analyzing genotypic controls (genomic DNA) and non-template controls in each individual 96-plate run.

The statistical significance in distributions of genotypes between the groups was tested using the chi-square function of the Epistat software (Tracy L. Gustafson, Round Rock, TX, USA).

3. Results

Table 1 shows that both SNPs were associated with diabetes risk in Finland and Sweden. The odd ratios (OR) associated with the protective genotypes of the two SNPs were of the same magnitude in the Finnish and Swedish populations. For both SNPs, the VNTR class III homozygote (TT for both SNPs) showed a stronger protective effect than the heterozygote. This was true for both of the populations. The differences between protective effects for the heterozygote and homozygote were significant in the combined data sets of the Finnish and Swedish samples ($p = 0.01$, OR $= 2.53$ for the -2221MspI, $p = 0.02$, OR $= 1.87$ for the -23HphI).

Both risk genotypes were significantly more common in Finland than in Sweden. This was true for both patients and controls, although the difference was slightly more pronounced among the controls ($p = 0.003$ and 0.0003 for patients and controls respectively in -2221MspI, $p = 0.0003$ and 0.0001 respectively in -23HphI). The combined homozygous protective genotype was twice as common in Sweden.

The combined -2221MspI – -23HphI genotypes and VNTR genotypes for patients and controls are shown in Table 2. As expected, the only genotype showing increased risk for T1D was VNTR class I/I genotype CC-AA. For both populations, the VNTR subclass IIIA homozygote showed the strongest protection for T1D; the odds ratios were 0.21 and 0.18 respectively for Finland and Sweden. The protective effect of the I/IIIA heterozygote was noticeably weaker than the effect of IIIA/IIIA in both populations and significantly weaker when the combined dataset was analyzed (Table 3). The difference between the effects of the subclass IIIB heterozygote and homozygote could not be tested reliably because the IIIB/IIIB genotype was rare in both populations, but the comparison in the combined datasets seems to suggest that subclass

Table 1
Genotype distributions of two single nucleotide polymorphisms in the insulin gene region among Finnish and Swedish children with type 1 diabetes (T1D) and controls (cont) and respective VNTR genotypes. P values are calculated for the difference in genotype distribution between cases and controls using predisposing CC or AA genotypes as a reference (OR = Odds ratio. 95% CI = 95% confidence interval)

Finland								
−2221MspI	VNTR genotype	T1D	%	cont	%	p-value	OR	95% CI
CC	I/I or I/III	515	84.8	418	68.9		1.0	
CT	I/III or III/III	88	14.5	174	28.7	<1.0E-6	0.41	0.30–0.55
TT	III/III	4	0.7	15	2.5	0.006	0.22	0.06–0.70
sum		607		607				
−23HphI		T1D	%	cont	%	p-value	OR	95% CI
AA	I/I	483	79.6	362	59.6		1.0	
AT	I/III	115	18.9	218	35.9	<1.0E-6	0.40	0.30–0.52
TT	III/III	9	1.5	27	4.4	0.0003	0.25	0.11–0.56
sum		607		607				
Sweden								
−2221MspI		T1D	%	cont	%	p-value	OR	95% CI
CC	I/I or I/III	272	76.2	454	59.8		1.0	
CT	I/III or III/III	80	22.4	261	34.4	7.8E-6	0.51	0.38–0.69
TT	III/III	5	1.4	44	5.8	0.0002	0.19	0.07–0.51
sum		357		759				
−23HphI		T1D	%	cont	%	p-value	OR	95% CI
AA	I/I	244	68.3	380	50.1		1.0	
AT	I/III	101	28.3	310	40.8	1.3E-6	0.51	0.38–0.68
TT	III/III	12	3.4	69	9.1	3.2E-5	0.27	0.14–0.53
sum		357		759				
Finland+Sweden								
−2221MspI		T1D	%	cont	%	p-value	OR	95% CI
CC	I/I or I/III	787	81.7	872	63.8		1.0	
CT	I/III or III/III	168	17.4	435	31.9	<1.0E-6	0.43	0.35–0.53
TT	III/III	9	0.9	59	4.3	<1.0E-6	0.17	0.08–0.36
sum		964		1366				
−23HphI		T1D	%	cont	%	p-value	OR	95% CI
AA	I/I	727	75.4	742	54.3		1.0	
AT	I/III	216	22.4	528	38.7	<1.0E-6	0.42	0.34–0.51
TT	III/III	21	2.2	96	7.0	<1.0E-6	0.22	0.13–0.37
sum		964		1366				

IIIB conveys a weaker protective effect than subclass IIIA (Tables 2 and 3). This was further demonstrated by the non-significant difference between the protective effects of I/IIIA and IIIA/IIIB and by the clear (although non-significant) difference between the effects of IIIA/IIIB and IIIA/IIIA (OR = 1.84) (Table 3).

4. Discussion

Our study confirms that type 1 diabetes risk is associated with homozygosity for the class I VNTR haplotypes within the insulin gene region. Heterozygotes carrying either IIIA (T-T) or IIIB (C-T) haplotypes with a class I haplotype both had a significantly lower risk of developing T1D than the class I homozygote in the Finnish and Swedish populations. As both class III haplotypes are characterized by a T allele at −23HphI, this

SNP is an optimal choice for the purpose of simple diabetes risk screening and the method we developed for defining the alleles offers an option for a rapid screening assay. The developed method applies the general principles we have used in high-throughput genetic tests for screening T1D susceptibility in the general population. These tests have been based on the amplification of polymorphic gene regions directly from blood spots on filter paper thus eliminating the laborious DNA extraction. This is followed by sequence-specific hybridization reactions performed in a microtitration plate format and a time-resolved fluorometry-based reading of hybridization reactions where the multiple lanthanide labels facilitate the simultaneous use of several probes in the same reaction [19,20]. We have earlier adopted this technology for the detection of the INS gene −2221MspI single nucleotide polymorphism [15]. To maximize the simplicity of the new assay for −23HphI,

Table 2
Combined genotypes for −2221MspI and −23HphI and their effect on diabetes risk in the Finnish. Swedish and combined populations. The VNTR genotypes are deduced from the −2221MspI – −23HphI haplotypes. P-values are calculated for the difference in genotype distribution between the T1D patients and controls using the predisposing CC-AA genotype as a reference (OR = Odds ratio. 95% CI = 95% confidence interval. ns = non-significant)

Finland								
Genotype	VNTR genot.	T1D	%	Controls	%	p-value	OR	95% CI
CC-AA	I/I	482	79.4	362	59.6		1.00	
CC-AT	I/IIIB	32	5.3	54	8.9	0.0006	0.44	0.27–0.72
CC-TT	IIIB/IIIB	1	0.2	2	0.3			
CT-AA		1	0.2	0	0			
CT-AT	I/IIIA	83	13.7	163	26.9	<1.0E-6	0.38	0.28–0.52
CT-TT	IIIA/IIIB	4	0.7	11	1.8	0.04	0.27	0.07–0.94
TT-AA		0	0	0	0			
TT-AT		0	0	1	0.2			
TT-TT	IIIA/IIIA	4	0.7	14	2.3	0.007	0.21	0.06–0.70
sum		607		607				
Sweden								
CC-AA	I/I	243	68.1	378	49.8		1.00	
CC-AT	I/IIIB	27	7.6	70	9.2	0.04	0.60	0.36-0.99
CC-TT	IIIB/IIIB	2	0.6	6	0.8	ns	0.52	
CT-AA		1	0.3	2	0.3			
CT-AT	I/IIIA	74	20.7	239	31.5	3.3E-6	0.48	0.35–0.66
CT-TT	IIIA/IIIB	5	1.4	20	2.6	ns	0.39	0.12–1.11
TT-AA		0	0	0	0.0			
TT-AT		0	0	1	0.1			
TT-TT	IIIA/IIIA	5	1.4	43	5.7	0.0001	0.18	0.06–0.49
sum		357		759				
Fin+Swe								
CC-AA	I/I	725	75.2	740	54.2		1.00	
CC-AT	I/IIIB	59	6.1	124	9.1	1.5E-5	0.49	0.34–0.68
CC-TT	IIIB/IIIB	3	0.3	8	0.6	ns	0.38	0.08–1.59
CT-AA		2	0.2	2	0.1			
CT-AT	I/IIIA	157	16.3	402	29.4	<1.0E-6	0.40	0.32–0.49
CT-TT	IIIA/IIIB	9	0.9	31	2.3	0.001	0.30	0.13–0.65
TT-AA		0	0.0	0	0.0			
TT-AT		0	0.0	2	0.1			
TT-TT	IIIA/IIIA	9	0.9	57	4.2	<1.0E-6	0.16	0.07–0.34
sum		964		1366				

Table 3
Statistical significance of differences between the protective effects of the class III VNTR genotypes in combined Finnish and Swedish dataset. P-values are calculated using a 2x2 contingency table (OR = Odds ratio. 95% CI = 95% confidence interval)

Fin+Swe			
VNTR genotype	p-value	OR	95% CI
I/IIIA vs IIIA/IIIA	0.02	2.47	1.15–5.50
I/IIIA vs IIIA/IIIB	ns	1.35	0.60–3.12
I/IIIB vs IIIB/IIIB	ns	1.27	
I/IIIB vs IIIA/IIIA	0.006	3.01	1.33–7.02
IIIA/IIIB vs IIIA/IIIA	ns	1.83	0.59–5.73

we used allele-specific probes labelled with different lanthanide chelates. This enables the detection of both alleles in one 96-plate well instead of two wells as in the previous method [15].

Conversely to some previous findings [1], our data suggest a stronger protective effect for the more com-mon subclass IIIA (PH) instead of IIIB (VPH). The effect appears also to be genotype-specific since the significance is due to the strong protective effect of the IIIA/IIIA genotype when it is compared to other geno-types containing the class III haplotypes. No signifi-cant or otherwise striking difference is found when the other class III genotypes are compared with each other. The significance of the differences seen in the protec-tive effects here can still be considered nominal, espe-cially if the p-values are corrected for multiple testing. Regardless of that, the same magnitude and direction of the protective effects seen in two independent popu-lations could indicate real effects and warrants further studies in bigger cohorts. The variation in the protec-tive effects of the different class III genotypes revealed by the subtyping of the class III lineages suggests that genotyping both −23HphI and −2221MspI could have

potential for added specificity and sensitivity in T1D risk estimation in the insulin gene region.

We also detected an unequivocal difference in the frequency of the risk and protective genotypes between the Finnish and Swedish populations although the risk associated with the analyzed genotypes was very similar in both countries. The significant difference in the frequencies of the risk and protective INS genotypes between Finland and Sweden might contribute to the known difference in the incidence of T1D between the two populations (43.9 for Finland, 25.7 for Sweden) [21], although definite claims cannot be made based on the results presented here.

Acknowledgements

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