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ABSTRACT

We elucidated the effect of four known T1D-susceptibility associated single nucleotide polymorphism (SNP) markers in three genes (rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS* and rs2476601 in *PTPN22*) on CpG site methylation of their proximal promoters in different lymphocyte subsets using pyrosequencing. The study cohort comprised 25 children with newly diagnosed T1D and 25 matched healthy controls. The rs689 SNP was associated with methylation at four CpG sites in *INS* promoter: −234, −206, −102 and −69. At all four CpG sites, the susceptibility genotype AA was associated with a higher methylation level compared to the other genotypes. We also found an association between rs12722495 and methylation at CpG sites −373 and −356 in *IL2RA* promoter in B cells, where the risk genotype AA was associated with lower methylation level compared to the AG genotype. The other SNPs analyzed did not demonstrate significant associations with CpG site methylation in the examined genes. Additionally, we compared the methylation between children with T1D and controls, and found statistically significant methylation differences at CpG −135 in *INS* in CD8+ T cells ($p=0.034$), where T1D patients had a slightly higher methylation compared to controls (87.3 ± 7.2 vs. 78.8 ± 8.9). At the other CpG sites analyzed, the methylation was similar. Our results not only confirm the association between *INS* methylation and rs689 discovered in earlier studies but also report this association in sorted immune cells. We also report an association between rs12722495 and *IL2RA* promoter methylation in B cells. These results suggest that at least part of the genetic effect of rs689 and rs12722495 on T1D pathogenesis may be conveyed by DNA methylation.

Introduction

Type 1 diabetes (T1D) results from immune-mediated damage of insulin-producing beta cells in the pancreas. CD4+ and CD8+ T cells and B cells are believed to have a pivotal role in this autoimmune response and many T1D susceptibility-associated single nucleotide polymorphisms (SNP) are located in genes expressed by these immune cells [1]. Over 70 genetic loci have been associated with T1D susceptibility: the HLA region with the strongest impact and many non-HLA SNP markers with smaller effects [2–4]. Unlike the HLA variants, relatively few non-HLA SNP markers reside within coding regions, and have a direct effect on protein structure, but rather appear to be enriched in enhancer regions [3] and may thus affect gene expression

levels [5]. This is suggested by the associations of SNPs with CpG methylation [6], which has an important role in gene expression regulation [7].

Research indicates that epigenetic mechanisms, such as DNA methylation, have a role in the pathogenesis of many autoimmune diseases [8,9] and there are also indications that disease-associated SNPs can have an effect on methylation in nearby regions [10]. In T1D, earlier studies have found associations between T1D-linked SNPs and CpG methylation both in wider analyses [11,12] and in targeted studies [13,14], where the affected CpG regions have often been situated close to the SNPs [12,15]. For example, associations have been reported between *INS* methylation and the rs689 SNP [11,12,14] and *IL2RA* methylation and 28 SNPs situated close by [13]. T1D-associated methylation

differences have also been observed in studies comparing T1D cases and controls [16,17]. These comparisons and SNP analyses have mostly been performed with samples such as whole blood, cord blood and PBMC that contain a heterogeneous mixture of immune cells. Since methylation patterns may be cell population-specific, these differences could also result from differences in immune cell frequencies within the samples [18].

In this study, we analyzed the association between four known T1D-susceptibility-associated SNP markers in three genes (rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS* and rs2476601 in *PTPN22*) and CpG site methylation in their proximal gene promoters in three immune cell populations implicated in T1D pathogenesis: B cells and CD8+ and CD4+ T cells. Outside the HLA region, *INS, PTPN22,* and *IL2RA* are among the most important T1D susceptibility genes [1]. Both *IL2RA* and *PTPN22* have an important role in regulating immune cell activation and TCR-signaling, whereas *INS* expression by the thymic epithelial cells during T cell selection shapes the T cell compartment by pruning autoreactive T cells. Outside the thymus, *INS* is only expressed in beta cells, where it is likely an important target for CD8+ T cells during autoimmunity. We also compared the methylation status of these three genes between immune cells from children with T1D and age- and sex-matched healthy controls.

Materials and methods

Study subjects

The study cohort comprised 25 case subjects with newly diagnosed T1D (mean age 7.5 ± 3.7; 1.6–14.5 years; sampled within 6 days after clinical diagnosis) and 25 healthy autoantibody-negative control subjects (mean age 7.3 ± 3.9; 1.1–14.4 years) matched based on HLA class II genotype, gender, age, and date of sampling. The T1D diagnosis was performed according to the criteria of the American Diabetes Association (ADA) [19]. Blood samples were collected as a part of the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study at the Turku University Hospital. For this project, T1D subjects were chosen based on the availability of a blood sample taken at the time of the T1D diagnosis and availability of a sample from a suitable control subject. All study subjects were male and all had HLA genotypes associated with increased risk of developing T1D: 28 subjects carried the HLA-DR3-DQ2/HLA-DR4-DQ8 genotype and 22 subjects HLA-DR4-DQ8/x genotype, where x was any haplotype other than HLA-DR3-DQ2 or HLA-DR4-DQ8. The T1D-associated autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GADA), and islet antigen-2 (IA–2A) were analyzed with specific radio-binding assays as previously described [20]. The samples were collected between 1999 and 2016 and the PBMCs isolated from blood by gradient centrifugation were stored in a viable state at −135 °C until analyses. The study was originally approved by the Ethics Committee of the Hospital District of Southwest Finland, followed by the Ethics Committee of the Hospital District of Northern Ostrobothnia, and written informed consent was provided by the families participating in the study.

Genotyping

HLA class II genotype for the major T1D risk alleles was determined, as previously described [21]. The subjects were also genotyped for four T1D-susceptibility-associated SNP markers in three genes: *IL2RA* (rs12722495, rs2104286), *INS* (rs689), and *PTPN22* (rs2476601). The SNP markers were genotyped using TaqMan SNP Genotyping Assays according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Isolation of B cells and CD4+ and CD8+ T cells

Frozen PBMC samples were thawed and the cells were rested overnight in a culture medium at +37 °C. Surface staining of the cells was performed the next day with antibodies to CD3 (APC-F750, Biolegend), CD4 (PE-Cy7, Biolegend), CD8 (FITC, Biolegend), CD20 (PE-CF594, BD), CD127 (APC, Biolegend), CD25 (PE, Miltenyi) and CD45RO (AF700, Biolegend). Dead cells were excluded by 7-AAD (Biolegend) staining. B cells (CD20+), CD8+ T cells (CD3 + CD8+) and CD4+ T cells (CD3 + CD4 + CD25lowCD127+) were isolated using flow cytometric sorting (FACSAria III, BD Biosciences, USA). After sorting the cell pellets were frozen and stored at −80 °C.

Isolation of genomic DNA and bisulfite conversion

DNA was extracted from the cell pellets with a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) and genomic DNA was then treated with EZ DNA Methylation-Gold Kit (Zymo Research Corporation, California, USA), according to the manufacturer's protocol.

Pyrosequencing

DNA methylation was examined with pyrosequencing at seven CpG sites within the *IL2RA* promoter (−459, −456, −373, −356, −272, −241 and −134), six CpG sites within the *INS* promoter (-234, −206, −180, −135, −102 and −69) and six CpG sites within the *PTPN22* promoter (-558, −546, −288, −164, −96 and −68). PCR- and pyrosequencing primers and assays were designed with the PyroMark Assay Design Software 2.0 (Qiagen, Hilden, Germany). Pyrosequencing was performed using a PyroMark Q24 pyrosequencing instrument (Qiagen) and results were analyzed with PyroMark Q24 Advanced software (Qiagen).

Briefly, after bisulfite conversion, DNA was PCR-amplified (primer sequences in Supplementary Table 1). Biotin-labeled single-stranded amplicons were subsequently purified with the PyroMark Q24 Vacuum Workstation (Qiagen) according to the manufacturer's protocol. After this, quantitative pyrosequencing was performed using the PyroMark Q24 pyrosequencing instrument (Qiagen). The methylation

percentage for each of the CpG sites was calculated using PyroMark Q24 Advanced software (Qiagen).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 27.0 software (Armonk, New York, USA). Methylation levels were compared between SNP genotypes, and cases and controls at variably methylated CpG sites. These were defined as CpG sites with more than a 5 percentage point difference between the second highest and second lowest value. This cut-off value was chosen based on earlier studies observing that quantitative differences as low as 5% can be reliably detected with pyrosequencing [22]. Methylation was compared between the different cell subsets using a non-parametric Kruskal-Wallis H test. Posthoc analyses were performed to examine differences between the groups. Methylation between the SNP genotypes, and cases and controls, was compared using the non-parametric Mann-Whitney U-test. The p values from the analyses were corrected for multiple comparisons using the Benjamini-Hochberg method. After correction, results with p values <0.05 were considered statistically significant. For the SNP analyses, the AT and TT genotypes in rs689 and the AG and AA genotypes in rs2476601 were grouped together. The genotype counts for the analyzed SNPs are shown in Supplementary Table 2.
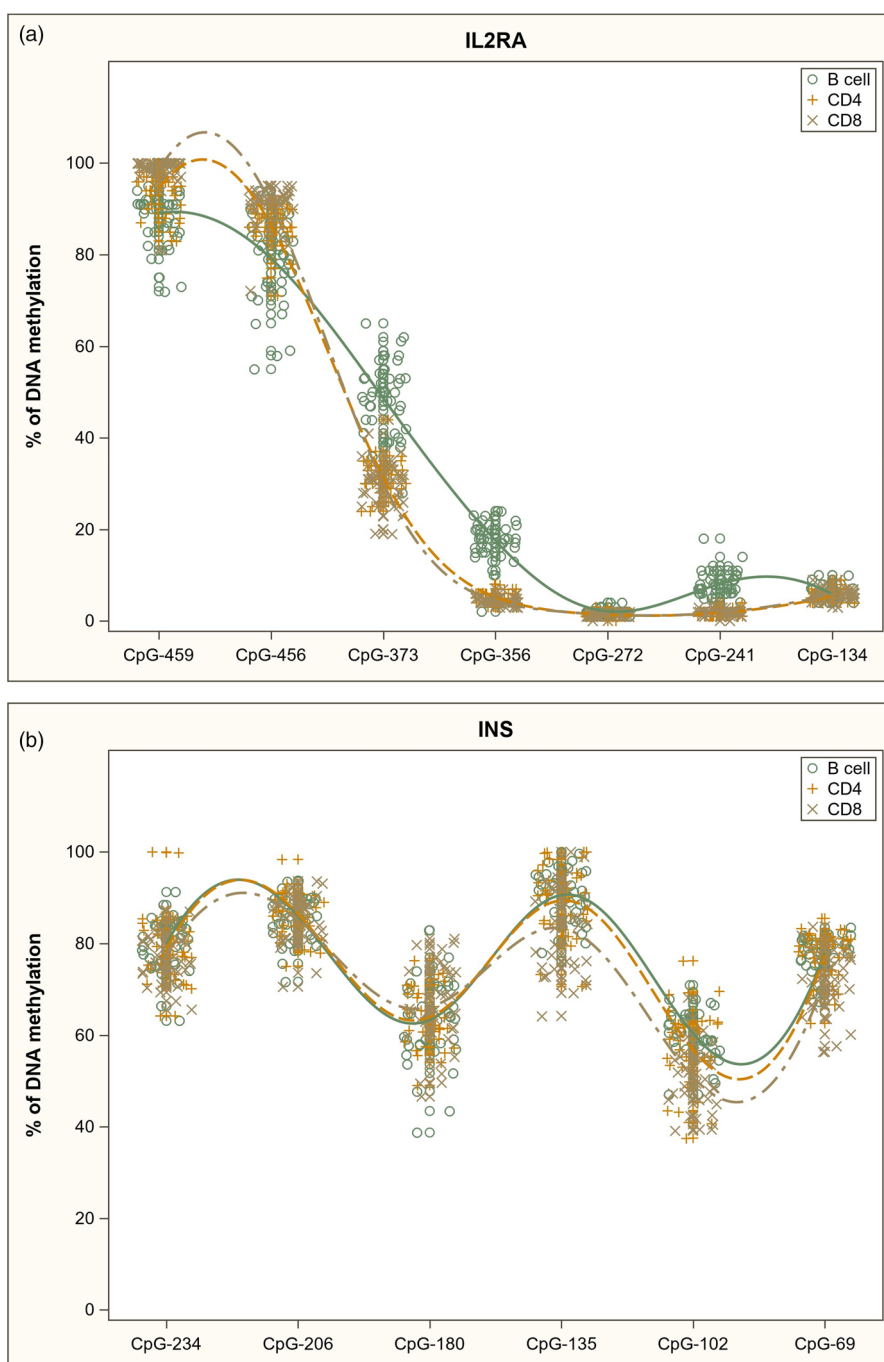

Figure 1. DNA methylation profiles in sorted immune cell subsets in analyzed gene promoters: a) *IL2RA*, b) *INS* and c) *PTPN22*.
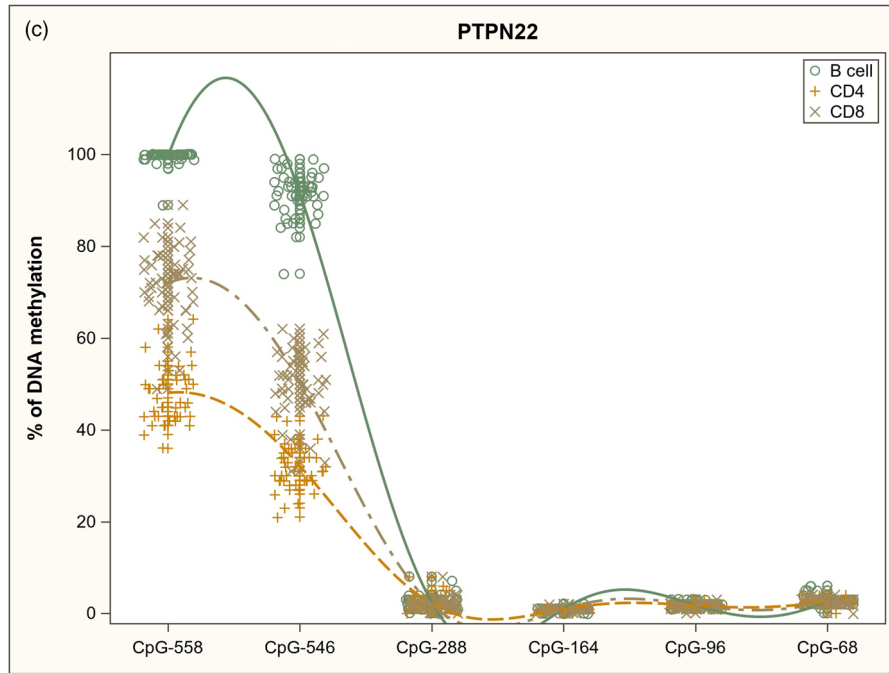
Figure 1. Continued.

Results

We used pyrosequencing to analyze DNA methylation in *IL2RA, INS* and *PTPN22* promoters (analyzed CpG sites are shown in Supplementary Figure 1) in three sorted immune cell populations, CD4+ and CD8+ T cells and B cells, in 25 case subjects with newly diagnosed T1D and in 25 healthy autoantibody-negative control subjects matched based on HLA class II genotype, age and date of sampling.

Cell population-specific DNA methylation patterns

DNA methylation patterns within the analyzed gene promoters in sorted immune cell subsets are shown in Figure 1. Within the *IL2RA* promoter (Figure 1a) methylation differed between the cell subsets at six out of the seven analyzed CpG sites: −459 ($p = 2.11E-15$), −456 ($p = 2.11E-15$), −373 ($p = 0.00E + 00$), −356 ($p = 0.00E + 00$), −272 ($p = 4.05E-4$) and −241 ($p = 0.00E + 00$), but the methylation differences were rather small, with only B cells clearly differentiated from the other subsets at three CpG sites: −373 (B cells 49% methylated, CD8+ T cells 30% and CD4+ T cells 31%), −356 (18%, 4% and 5%) and −241 (8%, 2% and 2%).

Similarly, within the *INS* promoter (Figure 1b), methylation differences between the cell subsets were small. Methylation differed between the subsets at three CpG sites: −135 ($p = 1.04E-2$), −102 ($p = 5.43E-6$), and −69 ($p = 2.04E-4$), where CD8+ T cells had only slightly lower methylation levels compared to the other subsets.

Within the *PTPN22* promoter (Figure 1c), methylation at CpG sites −558 ($p = 0.00E + 00$) and −546 ($p = 0.00E + 00$) varied considerably between all three cell subsets. By contrast at CpG sites −288, −164, −96, and −68 methylation was low in all cell subsets.

In summary, we observed cell subset-specific DNA methylation patterns in other analyzed gene promoters except for the *INS* gene where quite similar distributions in methylation were seen in all cell populations.

Influence of SNP genotypes on CpG methylation

We compared methylation levels between individuals carrying different T1D susceptibility-associated SNP markers at the specific promoter CpG sites where methylation variation in immune cell subsets was detected (Table 1). After correcting for multiple comparisons, we observed a statistically significant association between the rs689 SNP and four CpG sites within the *INS* gene promoter: −234, −206, −102 and −69. At CpG sites −234 and −206 the association was statistically significant in all analyzed subsets. At −102 the association was significant in CD4+ and CD8+ T cells but at CpG −69 only in CD4+ T cells. The susceptibility genotype AA was associated with higher methylation at all these CpG sites (Figure 2). We also found an association between rs12722495 and methylation at CpG sites −373 and −356 within the *IL2RA* promoter in B cells (Figure 2). At both CpG sites, the risk genotype AA was associated with lower methylation. The other analyzed SNPs did not demonstrate any statistically significant association with CpG site methylation in the examined genes. P values for all analyzed SNP-CpG-pairs are shown in Supplementary Table 3.

Association between T1D and CpG methylation

Finally, we compared methylation levels between patients with T1D and matched healthy controls at CpG sites where methylation variation in lymphocyte subsets was detected. Differentially methylated CpG sites and their methylation levels are shown in Table 2. We found a statistically

Table 1. Association between SNP markers and CpG site methylation: p values statistically significant before correcting for multiple comparisons, and the corrected p values.

SNP	Cell subset	Gene	CpG site	p value	Corrected p value (BH)
rs689	Bcell	INS	−234	4.00E-06	3.52E-05
	Bcell	INS	−206	6.22E-07	1.05E-05
	Bcell	INS	−102	0.014	5.13E-02
	CD4+	INS	−234	7.18E-07	1.05E-05
	CD4+	INS	−206	3.39E-07	1.05E-05
	CD4+	INS	−102	0.003	0.012
	CD4+	INS	−69	0.001	4.89E-03
	CD8+	INS	−234	2.00E-06	2.20E-05
	CD8+	INS	−206	1.06E-04	7.77E-04
	CD8+	INS	−180	0.042	1.41E-01
	CD8+	INS	−102	0.001	4.89E-03
	CD8+	INS	−69	0.045	1.41E-01
rs12722495	Bcell	IL2RA	−373	0.002	8.80E-03
	Bcell	IL2RA	−356	0.001	4.89E-03

significant methylation difference at CpG −135 in *INS* in CD8+ T cells, in which patients with T1D had slightly higher methylation compared to controls (87.3 ± 7.2 vs. 78.8 ± 8.9). At other analyzed CpG sites the methylation level was similar between the groups. P values for all analyzed CpG sites are shown in Supplementary Table 4. Since there was an association between the rs689 SNP and *INS* promoter methylation (Figure 2), we also separately compared the methylation level within these CpG sites between cases and controls carrying the susceptibility genotype (AA), but did not find any statistically significant differences after correcting for multiple comparisons.

Discussion

In this study, we examined the association between four known T1D-susceptibility-associated SNP markers in three genes (rs12722495 and rs2104286 in *IL2RA*, rs689 in *INS,* and rs2476601 in *PTPN22*) and CpG site methylation in their proximal gene promoters. We also compared methylation between patients with T1D and matched healthy controls. The methylation analysis was carried out in sorted immune cell populations to avoid potential issues with heterogeneous samples, such as PBMCs. Analyzing a mixture of different cells can hide small methylation changes within the subsets or the methylation changes could result from differences in cell subset frequencies rather than differences within the subsets. We found an association between the rs689 SNP marker and DNA methylation at four CpG sites within the *INS* promoter: −234, −206, −102, and −69. At all four sites, the susceptibility genotype AA was associated with higher methylation. We also found a T1D-associated methylation difference at CpG −135 in *INS* in CD8+ T cells, where T1D patients had slightly higher methylation than controls.

The association between rs689 SNP and *INS* promoter methylation was first discovered by Fradin et al. [14] who observed that the risk genotype was associated with higher methylation at four CpG sites (−206, −180, −102, and −69) in whole blood cell samples. This association was later confirmed in other studies, which also reported that, in addition to the proximal promoter, rs689 SNP seems to be associated with methylation at CpG sites further upstream

[11,12]. There is also evidence for T1D-specific methylation changes in the *INS* promoter [11,14,23]. Fradin et al. [14] found a methylation difference between patients with T1D and controls at four CpG sites including CpG −135, which was observed to be differentially methylated also in our study. Carry et al. later confirmed the result for two of these sites and one additional CpG site [11]. Interestingly, similar methylation changes have also been observed in blood [14] and islet cells [24] from patients with type 2 diabetes. Apart from the CpG site −135 in CD8+ T cells, we did not see any statistically significant differences between our cases and controls, but this difference compared to earlier reports could be due to our smaller study size.

Collectively the results from the current study and previous work indicate that there is an association between rs689 and *INS* gene methylation and also between T1D status and *INS* methylation but whether these associations have any functional role is still an open question. We know that demethylation at these CpG sites within the *INS* promoter is associated with *INS* gene expression. Kuroda et al. examined nine CpG sites in the human *INS* promoter and showed that they are unmethylated in the islet cell fraction of pancreas and methylated in the non-islet fraction [25]. They also showed experimentally that methylation of these sites represses *INS* expression. Other studies have later confirmed such an inverse correlation [24,26] and observed other sites upstream that could also have an effect on the expression [24,27].

INS is expressed in pancreatic beta cells but also in the thymus during T-cell selection. Decreased expression of *INS* by thymic epithelial cells in individuals with the risk genotype could lead to autoreactive T cells escaping negative T-cell selection and thereby conferring a higher risk of beta-cell autoimmunity. The rs689 SNP is in linkage disequilibrium (LD) with the 5′ variable number tandem repeat (VNTR) polymorphism situated in the non-coding region in the *INS* gene [28,29], and the VNTR polymorphism has been associated with T1D risk and the susceptibility variant with lower expression of *INS* in the human thymus [30].

We also found an association between the rs12722495 risk genotype and decreased methylation at CpG sites −373 and −356 within the *IL2RA* promoter in B cells. The rs2104286 risk genotype was not associated with *IL2RA* methylation and we did not find any T1D-associated

Association between rs689 genotypes and CpG methylation
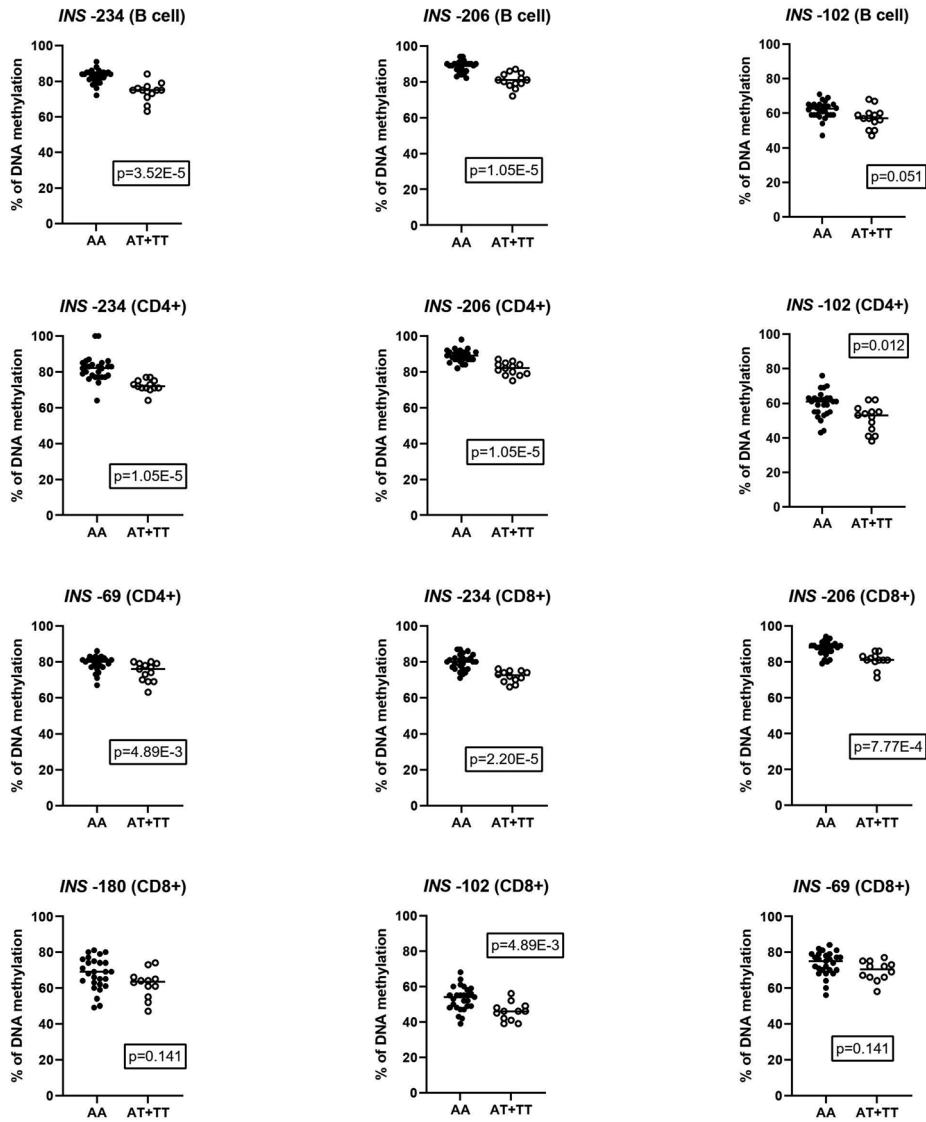
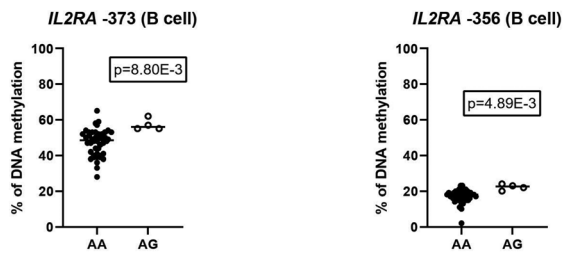## Association between rs12722495 genotypes and CpG methylation

Figure 2. CpG sites where DNA methylation is associated with rs689 and rs12722495 genotypes.

methylation changes within the *IL2RA* promoter. Hypomethylation within the *IL2RA* promoter has earlier been associated with multiple sclerosis (MS) [31] and autoimmune thyroid disease (AITD) [32]. T1D-associated methylation differences in *IL2RA* have also been found at CpG sites −456 and −373, but more importantly, it was discovered that sixteen T1D-associated SNPs, including rs2104286, showed an association with methylation at CpG site −373 [13]. Promoter methylation does seem to correlate inversely with *IL2RA* expression [31,33] and collectively, these results indicate that changes in DNA methylation could convey at least part of the effect of the rs12722495 SNP and that particularly methylation at CpG site −373 may be important.

Table 2. DNA methylation levels (%) in T1D patients and controls: results with significant p-values before correction, and their p values after correcting for multiple comparisons.

Cell subset	Gene	CpG site	T1D patients	Controls	p-value	Corrected p values (BH)
CD4+	*INS*	−234	81.2 ± 8.0	76.1 ± 6.2	0.047	0.228
CD8+	*INS*	−234	79.0 ± 4.7	75.6 ± 5.7	0.046	0.228
CD8+	*INS*	−206	87.0 ± 4.0	83.5 ± 5.7	0.032	0.218
CD8+	*INS*	−180	69.5 ± 7.6	62.1 ± 8.9	0.017	0.145
CD8+	***INS***	**−135**	**87.3 ± 7.2**	**78.8 ± 8.9**	**0.001**	**0.034**
CD8+	*INS*	−102	53.6 ± 5.4	47.6 ± 7.5	0.003	0.051
CD8+	*INS*	−69	74.8 ± 4.6	69.2 ± 7.2	0.009	0.102

We also analyzed methylation within the *PTPN22* promoter but we did not find any methylation differences between rs2476601 genotypes, or between T1D cases and controls. There have only been a few studies examining *PTPN22* methylation in a targeted manner and none regarding T1D status or rs2476601 genotype but *PTPN22* promoter hypermethylation has been observed in Hashimoto thyroiditis [34] and psoriasis [35]. Methylation in the promoter at CpG sites −288, −164, −96, and −68 has previously been reported to correlate inversely with *PTPN22* expression [35] and now our findings indicate that DNA methylation levels at CpG sites −558 and −546 in *PTPN22* promoter distinguish CD4+ and CD8+ T cells and B cells from each other and may indicate a transcription factor binding site in the promoter.

Broader methylation arrays combined with GWAS data have also found associations between T1D associated SNPs and DNA methylation [11,12,15]. Using cord blood from newborn infants with risk genes for T1D, Kindt et al. [15] found CpG sites where levels of methylation were associated with SNP mutations from all analyzed T1D susceptibility genes including *INS*, *C1QTNF6*, *CDK4*, *CLEC16A*, *CTSH*, *PFKFB3*, *PSMG2*, *RGS1*, *SULT1A2* and *UBASH3A*. In another GWAS study, Ye et al. [12] found 96 SNP-CpG pairs and in 5 of these loci, *ITGB3*, *AFF3*, *PTPN2*, *CTSH*, and *CTLA4*, DNA methylation seemed to mediate the genetic risk by affecting gene expression [12]. Later Carry et al. [11] examined associations between methylation and 13 T1D-associated SNPs using peripheral blood samples from 83 cases and 83 controls from the DAISY study. The Infinium Human Methylation 450K and EPIC beadchip data revealed 10 SNP-CpG pairs associated with two novel gene loci, *GSDMB* and *IL27*, and two loci discovered earlier: *C1QTNF6* and *INS* [11]. These results indicate that DNA methylation could mediate at least part of the effect of T1D susceptibility genes.

In addition to the effect of genetic variation, there are also other inter-individual variations in DNA methylation possibly caused by environmental factors. The role of DNA methylation in T1D development has been assessed in several studies and methylation changes preceding the T1D diagnosis [16,17] or autoantibody-positivity [36] have been reported. These studies have been conducted with either whole blood or cord blood [16], or with immune cell subsets purified from peripheral blood [17,36]. Of note, many discovered differentially methylated regions were cell subset-specific [36].

In conclusion, we observed an association between rs689 and *INS* promoter methylation in sorted immune cells. We also found an association between rs12722495 and *IL2RA* promoter methylation in B cells. These findings are in line with earlier studies using whole blood and cord blood samples. Together with earlier reports, our current study suggests that at least part of the effect of rs689 and rs12722495 on T1D pathogenesis may be conveyed by DNA methylation. The cell subset-specific DNA methylation patterns we observed, underline how important it is to carefully consider the analyzed material in DNA methylation studies and to use sorted cell subsets when possible. Future case-control studies should also take genetic variation into account to make sure any methylation differences between cases and controls are not due to different distribution of T1D-risk genotypes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The datasets used and/or analyzed in the present study are not readily available due to data privacy regulations. Requests to access the datasets should be directed to DIPP Steering Committee, johanna.lempainen@utu.fi.

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