



Differences in promoter DNA methylation and mRNA expression of individual alleles of the HLA class II DQA1 gene



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ABSTRACT

Objectives: Extensive polymorphism of HLA class II genes is not restricted to the coding region of the gene. It extends also to the linked promoter region, where it forms the basis for different levels of individual allele's expression. Differential expression of HLA class II alleles can shape an immune response and influence the risk of developing autoimmune disease. In addition to genetic variability, variation in epigenetic modifications, including DNA methylation, can be another cause of the uneven expression of individual alleles. We aimed to analyze the DNA methylation of promoter sequences and the levels of expression of individual DQA1 gene alleles, interallelic variation of these two characteristics and the relationship between them.

Methods: The 60 healthy donors included into study were HLA-DRB1, HLA-DQB1 and HLA-DQA1 genotyped using PCR-SSP. Genomic DNA was treated by sodium bisulfite and the target segment in the HLA-DQA1 gene promoter was PCR amplified. PCR product was cloned into *Escherichia coli* and individual clones were sequenced. Transcripts of individual DQA1 alleles in peripheral blood leukocytes were quantified by Real-Time PCR.

Results: In this study, we have described detailed DNA methylation profile of promoter area of DQA1 gene alleles. The overall promoter methylation is increased for DQA1*02:01 and DQA1*04:01 alleles, on the other side, DQA1*05:01 allele shows decreased methylation level. Our results suggest that there are only minor interindividual differences in DRA-normalized expression level of specific allele. Furthermore, expression levels of individual alleles followed DQA1*03 > *01:03 (in DRB1*13-DQA1*01:03-DQB1*06:03 haplotype) > *01:01, *05:05, and DQA1*03 > *02:01 > *05:05 hierarchy. The statistically significantly most expressed allele, DQA1*03, comprises part of DQ8 molecule, which is commonly linked to autoimmune diseases. A clear relationship between promoter DNA methylation and mRNA expression level of the DQA1 gene could not be identified.

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1. Introduction

Human leukocyte antigen (HLA) class II molecules play a key role in the immune response by presenting peptides to the antigen receptor of CD4+ T lymphocytes. They participate in the selection of T cell repertoire in the thymus and their expression on the surface of antigen presenting cells (APC) is essential for the initiation of the adaptive immune response [1]. There are three isotypes of class II molecules, HLA-DR, HLA-DQ and HLA-DP. Each isotype consists of two noncovalently associated transmembrane chains, α and β ,

whose extracellular parts together make up an antigen-binding site [2].

HLA class II genes are highly polymorphic (with the exception of HLA-DRA), with tens to hundreds of variants known for each gene. Most of the variation is concentrated in the region coding for peptide-binding groove, and as a result, each class II heterodimer presents a different spectrum of peptides [2]. However, sequence differences are not restricted to coding regions; they are also abundant within the promoters. Allelic variants of promoter are known for HLA-DRA [3], DRB1 [4], DQA1 [5,6], and DQB1 [7] genes. Special notation, QAP, is used to refer to promoter alleles of HLA-DQA1 gene [8].

Different HLA class II allelic variants are often either positively or negatively associated with various autoimmune diseases. Since the immune response can be shaped by the amount of class II molecules on the cell's surface [9–11], this predisposition or protection to autoimmunity can be carried not only by polymorphisms in the

Abbreviations: QAP, allele; DQA1, promoter allele.

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coding region that affect the shape of peptide-binding groove, but also by factors influencing the gene's expression. As HLA expression is regulated primarily on the level of transcription [1], the polymorphisms in promoter region are of particular importance.

The promoter polymorphism is known to be functional and translates into variable levels of expression of HLA-DQA1 [12–15], HLA-DQB1 [12] and HLA-DRB1 [16] alleles. However, a consensus on the expression hierarchy was not always reached and sequence diversity in the promoter area alone is not able to fully explain variability in the gene's expression.

As a change in DNA sequence can translate into a change in the epigenetic state [17,18] and epigenetic changes at promoter are considered to be a prerequisite for successful gene expression [19–21], we decided to inspect epigenetic polymorphism at the promoter region of HLA class II genes as another possible cause of their differential expression. In this study we analyzed the DNA methylation status of individual DQA1 promoter alleles and the mRNA expression level of the linked DQA1 gene alleles. Interallelic differences in DNA methylation and mRNA expression were both found, however, no correlation between these two characteristics was revealed.

2. Materials and methods

2.1. Subject of the study

Our test group consisted of 60 healthy volunteers of European descent, 19–39 years old. This age restriction of individuals included into the group was chosen to limit the possible confounding effect of age on DNA methylation [22].

The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki. This study was approved by The Ethical Committee of the Third Faculty of Medicine of Charles University in Prague.

2.2. DQA1, DRB1, and DQB1 genotyping

Genomic DNA from peripheral leukocytes was extracted using salt-extraction (ethanol-precipitation) method (modified Miller et al. [23]) and diluted to the concentration of 30 ng/ μ l. HLA-DQA1 typing was performed by polymerase chain reaction with sequence-specific primers (SSP-PCR) using Olerup SSP™ HLA-DQA1 typing kits (Olerup SSP AB, Stockholm, Sweden). HLA-DR and HLA-DQB1 were genotyped using Olerup SSP™ HLA-DR low and Olerup SSP™ HLA-DQ low typing kit followed by allele-specific Olerup SSP™ HLA-DQB1 typing kits according to the manufacturer's instructions. PCR products were identified by 2% agarose gel electrophoresis.

2.3. DNA methylation analysis

2.3.1. Bisulfite sequencing

Genomic DNA from peripheral leukocytes was extracted using a salt-extraction method and converted by sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol.

Three independent reactions were employed to amplify the target segment in the HLA-DQA1 gene promoter region from bisulfite-treated genomic DNA. Reaction mix for nested PCR reaction1/reaction2 consisted of 1xTaq buffer with $\text{NH}_4(\text{SO}_4)_2$, 6 mM/4 mM MgCl_2 , 0.5 mM dNTP mix, 0.8 μ M of each primer, 1 U/25 μ l of Taq and 1 μ l/1.5 μ l of DNA solution, and cycling conditions were 95 °C for 5 min, followed by 40/50 cycles of 95 °C for 1 min, 56 °C/65 °C (55 °C/57 °C for the DQA1*04:01 allele) for

1.5 min, 72 °C for 1 min, and finally 72 °C for 10 min. Primer properties are shown in Supplemental Table A.1.

After finishing, PCR amplification triplicates were mixed and cloned into *E. coli* XL-1 Blue. Sequencing of individual clones was performed (Macrogen, Seoul, Korea) and sequences obtained were processed using BioEdit software.

2.3.2. Analysis of DNA methylation

Damaged sequences, sequences that recombined during PCR amplification, and sequences where bisulfite conversion efficacy was smaller than 95% were discarded. The methylation status of the remaining 213 DQA1 promoter sequences from 35 individuals was analyzed. Out of the 213 sequences, 182 covered whole promoter region studied and 31 lacked methylation data for one or more methylation sites within the region. To analyze site specific DNA methylation, data from 182 complete sequences and data from the informative CpG sites of 31 incomplete sequences were used. To analyze overall methylation, data from 182 full-length sequences were used.

Overall methylation, i.e. the number of methylated cytosines in CpG context per sequence, of individual DQA1 promoter alleles was compared by two-tailed Mann-Whitney non-parametric test with 95% confidence interval. To compensate for multiple comparisons, the Bonferroni correction was used (10 alleles tested, $P_{\text{corrected}(c)} = P_{\text{uncorrected}(un)} \times 10$).

The differences in individual CpG sites' methylation between alleles were analyzed by a two-tailed Fisher's exact test with a 95% confidence interval and a significance level of $\alpha = 0.05$ (RRs with 95% CI were also calculated). With regard to the non-identical number and position of CpG sites in individual alleles, for each site, only the data of alleles containing a particular CpG site (Fig. 1) were compared. Obtained p-values were subjected to the Bonferroni correction (11 alleles tested, $p_c = p_{un} * 11$).

2.4. Expression analysis

2.4.1. Primers and probes

Due to a high polymorphism of the target region, assays were designed to quantify whole allele groups instead of individual alleles (DQA1*01, *02, *03, *04, *05 assays). "DQA1 total" assay was used to quantify total DQA1 mRNA irrespective of alleles present, "DRA" assay targeting the HLA-DRA gene was used as an endogenous control, and assay amplifying an intronic segment of the DQA1 gene "DQA1 intron" was used to determine genomic DNA contamination of our samples. The design of all PCR primers and fluorogenic probes (synthesized by IDT, Coralville, Iowa, USA) was based on primers and probes used by Fernandez et al. [14] and modified according to the published sequences (<http://www.ebi.ac.uk/ipd/imgt/hla/align.html>). Sequences and properties of all primers and probes are listed in Supplemental Table A.1.

2.4.2. RNA extraction, cDNA synthesis and quantification

Total RNA was extracted from PBMC using QIAamp RNA Blood Mini Kit (Qiagen). Total RNA was reverse transcribed with random hexamer primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, USA). Wherever possible, RNA was isolated from the same blood draw as DNA isolated for bisulfite analysis.

All PCR amplifications were performed in triplicate and contained 200 nM of probe, 300 nM of each primer and 1x Gene Expression Master Mix (Applied Biosystems). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. For quantification, an Applied Biosystems 7500 Fast Real-Time PCR System was used.

Outlying values from each triplicate were omitted from further analysis. Values obtained for allele-specific assays were corrected

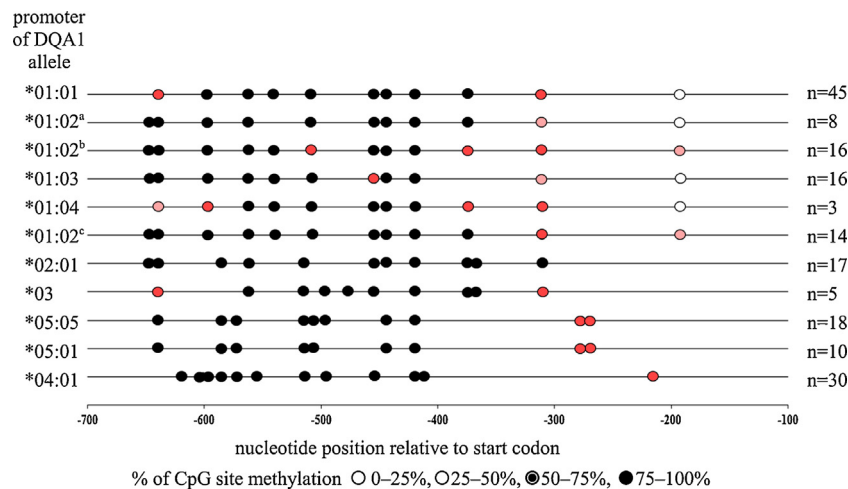


Fig. 1. CpG methylation status of individual CpG sites of DQA1 promoter alleles. According to the polymorphisms present on the sequencing read, sequences were classified into appropriate allelic groups.

The matrix represents an amplicon that contains 9–12 CpG sites (number depends on promoter allele identity) obtained for 500 bp region overlapping promoter of the DQA1 gene. Each colour-coded circle within the matrix represents one CpG site and its colour indicates the percentage of sequences that have the cytosine at the given site methylated. Unmethylated-low methylated sites are indicated by open circles. No interallelic difference in methylation of individual CpG sites was found to be significant after Bonferroni correction.

n – number of sequences analyzed.

^aLinked with DQA1 promoter allele QAP 1.2K.

^bLinked with DQA1 promoter allele QAP 1.2L.

^cLinked with DQA1 promoter allele QAP 1.4.

according to the assays efficacy and subsequently according to the genomic DNA content of the sample to reflect proportions in the RNA component of the sample only (see Section 2.4.4).

2.4.3. Analysis of allele-specific mRNA expression

Relative expression of DQA1 alleles was determined with reference to the amount of HLA-DRA mRNA. To ensure reliability of results, only samples where (allele 1 + allele 2 mRNA)/(total DQA1 mRNA) belonged into the <0.85; 1.15> interval were included into the subsequent analyses. Relative expression between all possible pairs of alleles was compared by a two-tailed Mann-Whitney test with 95% CI. All *p*-values were subjected to the Bonferroni correction for multiple comparisons (9 alleles tested, $p_c = p_{un} \cdot 9$).

Out of 43 subjects included into the expression study, 4 were heterozygous for the DQA1 alleles recognized by the same allele-specific assay, and therefore expression of individual alleles in these subjects could not be analyzed. In 4 homozygous individuals present in our sample, both DQA1 gene copies were considered to express mRNA equally, therefore the detected allele mRNA level in homozygotes represented double the amount of mRNA from a gene copy and was divided by 2. In the remaining 35 heterozygous individuals, altogether 22 allelic combinations were found.

Two DQA1*03 group alleles were found in our set—DQA1*03:01 and *03:03 (as a part of two different DRB1*04-DQA1*03-DQB1*03 haplotypes), however, since both these alleles are linked to the same promoter allele, QAP 3.1, their expression was analyzed together as a DQA1*03 allele group.

2.4.4. Detection of genomic DNA contamination, assay specificity, and assessment of relative amplification efficiencies

The content of genomic DNA in the samples was determined by an assay amplifying an intronic segment of the DQA1 gene [14]. Genomic DNA contamination was found to be 0–14% (mean $5.2 \pm 3.6\%$).

Specificity of each allele-specific assay was verified. The amount of off-target amplification was found to be less than 0.02% of target alleles amplification (=at least 12 cycle difference) for all assays used.

To determine the efficacy of allele-specific assays, DQA1-total assay has been assigned efficacy $E_{total} = 1$, and mean Ct of DQA1-total assay was compared with mean Cts of DQA1*01, *02, *03, *04, *05 assays in all available individuals homozygous for given DQA1 allele. There were no DQA1*04 homozygotes in our experimental set, therefore, the DQA1*04 assay efficacy was calculated from the data of individuals heterozygous for this allele and for the allele with already assessed efficacy. Only triplicates with overall SD < 0.1 were included into efficacy calculations. Efficacy of assays amplifying DQA1*01, *02, *03, *04 and *05 allele groups was found to be 1.02, 0.98, 0.97, 0.98 and 0.95, respectively.

DRA assay efficacy (E_{DRA}) was determined by using 10^0 to 10^{-4} serial dilutions of input cDNA as a template for amplification in both, DRA and DQA1 total assays. Standard curves were constructed, E_{total} was assigned a value of 1, and E_{DRA} relative to E_{total} was assessed by comparing the slopes. E_{DRA} was found to be the same as E_{total} and therefore was also assigned a value of 1.

3. Results

3.1. mRNA expression of DQA1 alleles

We analyzed mRNA expression data from 43 individuals. Data included 10HLA-DQA1 alleles linked with 12 different promoter alleles. Each DQA1 allele was linked to one promoter allele, except DQA1*01:02 that was linked to 3 different DQA1 promoters, QAP 1.2K, QAP 1.2L and QAP 1.4. DQA1 alleles and their linked promoter alleles (QAP) are listed in Table 1 as a part of the respective HLA class II haplotype [8]. Because of a low number of sequences obtained (1, 2 and 1, respectively), expression data of DQA1*01:02 (QAP 1.2K linked), DQA1*01:04, and DQA1*01:05 alleles were not included into the analysis. Average mRNA expression levels of individual alleles normalized to DRA varied from 0.07 for DQA1*05:05, through 0.13 for DQA1*01:02 (QAP 1.4 linked) as a second most expressed allele, to 0.23 for DQA1*03. DQA1*03 alleles were significantly overexpressed compared to most other alleles (DQA1*01:01, *01:02 (QAP 1.4 linked), *01:03, *02:01, *05:01, *05:05), DQA1*05:05 allele was expressed less than DQA1*01:03,

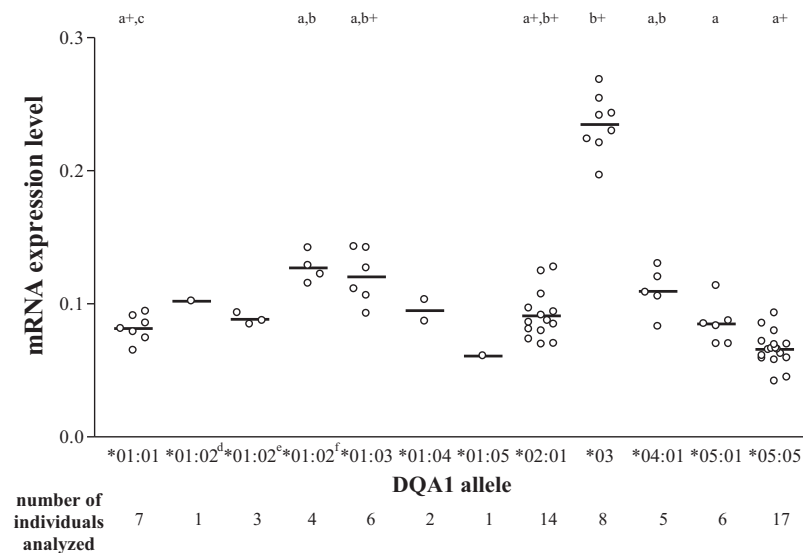


Fig. 2. mRNA expression levels of DQA1 alleles. Expression of DQA1 alleles was determined by qRT-PCR with allele-specific primers and the mean of 3 independent measurements was used for the analysis. Each point in the graph represents normalized value for 1 individual and 1 allele. The horizontal line indicates mean relative expression of the allele.

^{a(a+)} Allele expression significantly, $p < 0.05$ (highly significantly, $p < 0.005$) lower than that of DQA1*03 allele.

^{b(b+)} Allele expression significantly, $p < 0.05$ (highly significantly, $p < 0.005$) higher than that of DQA1*05:05 allele.

^c Allele expression significantly lower than that of DQA1*01:03 allele.

^d Linked with DQA1 promoter allele QAP 1.2K.

^e Linked with DQA1 promoter allele QAP 1.2L.

^f Linked with DQA1 promoter allele QAP 1.4.

Table 1

Analyzed DQA1 and DQA1 promoter (QAP) alleles as a part of DRB1-QAP-DQA1-DQB1 haplotype.

DRB1*	QAP ^a	DQA1*	DQB1*
01	1.1	01:01	05:01
16	1.2K	01:02 ^b	05:02
15	1.2L	01:02 ^b	06:02
13	1.3a	01:03	06:03
14	1.3b	01:04	05:03
10	1.3b	01:05	06:02
13	1.4	01:02 ^b	06:04, 06:09
07	2.1	02:01	02:02, 03:03
04	3.1	03	03, 02:02
11, 12	4.1A	05:05	03:01
03	4.1B	05:01	02:01
08	4.2	04:01	04:02

^a The available nomenclature of DQA1 promoter alleles (QAP) does not distinguish all alleles present in our samples, so we split some of the existing alleles into 2 groups and use our own marking to denote them. Based on the sequence differences, allele QAP 1.2 was split into groups QAP 1.2K and QAP 1.2L, QAP 1.3 into groups QAP 1.3a and 1.3b, and QAP 4.1 into QAP 4.1A and 4.1B. These “new” alleles differing in their sequence were also parts of different HLA-haplotypes.

^b Allele DQA1*01:02 is associated with 3 different promoters (in 3 different haplotypes).

*02:01, *04:01 alleles, and DQA1*01:03 allele was expressed more than DQA1*01:01 allele (Fig. 2, Supplemental Table A.2).

To analyze the differences in more detail, we calculated ratio between mean expression levels of the alleles. Mean expression ratio of alleles that proved to differ significantly ranged from 1.4 (DQA1*02:01:DQA1*05:05) to 3.6 (DQA1*03:DQA1*05:05) (Supplemental Table A.3). As a next step, we examined the allele expression ratio in the heterozygous individuals (Supplemental Table A.4). In heterozygotes, DQA1*03 allele was always expressed more than the other allele, on the other side DQA1*05:05 allele was always the less expressed one. Only exception was observed in single individual, where DQA1*01:05 allele was expressed slightly more than *05:05 allele (expression ratio 1.09). In single heterozygous individuals we observed distinct DQA1*01:03 > DQA1*02:01,

*05:01, and DQA1*01:02 (QAP 1.4 linked) > DQA1*05:01 relationship (allele ratio 1.3–1.7); and less pronounced (allele ratio close to one) DQA1*02:01 > DQA1*01:02 (QAP 1.2L linked), and DQA1*02:01 > DQA1*05:01 relationship. As these observations were done in single individuals only, no further conclusions regarding the hierarchy of allele’s expression could be made.

We also wondered whether and how the presence of specific DQA1 alleles in an individual’s genotype affects total DQA1 mRNA expression. From the analyses described above, it seems that an allele’s mRNA expression level tends to stay the same irrespective of the identity of the other allele present in a heterozygous combination. In this case, one should be able to calculate the total relative DQA1 mRNA level in the subject only by adding up the known values of a relative expression of the alleles carried by the subject. Indeed, we observed that amount of total relative DQA1 gene mRNA seen in individual samples (Supplemental Table A.5) followed the “theoretical” DQA1 total relative mRNA level, which was calculated as a sum of mean relative expression of alleles present in a sample (Supplemental Table A.6).

3.2. DNA methylation of HLA-DQA1 promoter region

Methylation status of 213 HLA-DQA1 promoter (QAP) sequences from 35 individuals was analyzed. Out of these 35 subjects, mRNA expression data were available for 18 individuals. Out of them, 14 had both expression and DNA methylation analyses done on the RNA and DNA isolated from the same blood draw.

3.2.1. Allele-specific methylation of individual CpG sites—methylation of individual CpG sites does not differ between promoter alleles

The only difference in DNA methylation of individual CpG sites found to be significant after correction was between DQA1*01:01 promoter and DQA1*01:02 (QAP 1.2L linked) at site –508 ($p_{un} = 0.0043/p_c = 0.0473$, RR CI = 0.9436 to 10.57). However, RR CI contained value 1 and therefore we could not reject the possibil-

ity that the association observed is only due to chance. No other differences in DNA methylation of any CpG site between any 2 alleles were observed (data not shown). The average methylation of individual CpG sites is shown in Fig. 1.

3.2.2. Overall methylation of individual alleles—the most methylated alleles are DQA1*02:01 and *04:01

Because of the low number (3) of DQA1*01:04 promoter allele sequences, we did not include it into methylation analysis.

Promoters of DQA1*02:01 and DQA1*04:01 were significantly more methylated than most of the other alleles (DQA1*01:01, *01:02—QAP 1.2K linked, *01:03, *05:01, *05:05); promoter of DQA1*04:01 was also methylated more than promoter of DQA1*03, and methylation of DQA1*01:02 (QAP 1.4 linked) promoter was higher than that of DQA1*01:01. Overall methylation ranged from 7.6 methylcytosines on average per promoter for DQA1*01:03 allele to 10.5 methylcytosines per promoter in DQA1*02:01 and 10.8 methylcytosines in DQA1*04:01. Overall methylation of individual alleles is depicted in Fig. 3, results of statistical analysis are shown in Supplemental Table A.7.

4. Discussion

4.1. mRNA expression of the HLA-DQA1 alleles

According to our findings, the relationship between mRNA expression levels of DQA1 alleles is DQA1*03 > *01:03 > *01:01, *05:05, and DQA1*03 > *02:01 > *05:05, with the average mRNA expression level of DQA1*03 alleles being approximately 2–4 times higher than the average mRNA expression of any other allele. In the heterozygous individuals present in our study sample, DQA1*03 allele was always overexpressed as compared to any other allele. In contrast, DQA1*05:05 allele was always the less expressed one (with exception of single individual, in whom *05:05/*01:05 ratio was found to be 1.09).

An expression hierarchy observed in peripheral blood cells by Donner et al. (DQA1*03 > *01 > *02:01 > *05) [13] and Maffei et al. (DQA1*03:01 > *05:01) [24], and in peripheral blood mononuclear cells by Britten et al. (DQA1*03, *01:03 > *05:01) [12] is in agreement with our results. Only study of Fernandez et al. found the expression of DQA1*04 allele to exceed expression of the other alleles [14]. This discrepancy is most probably caused by not considering differences in efficacy of individual assays. In conclusion, there seems to be an agreement on highest expression of DQA1*03, and lowest expression of DQA1*05 group of alleles.

Our results suggest that relative mRNA level of particular DQA1 allele does not show much interindividual variability, tends to remain constant for a particular allele, and is not dependent on the second allele present in heterozygous individual. If this is true, then the total relative amount of DQA1 mRNA (which is the sum of relative DQA1 mRNA levels of individual alleles) should differ between individuals in accordance with the alleles an individual carries. It was indeed observed that the sum of relative DQA1 allele expression in heterozygotes (Supplemental Table A.5) paralleled theoretical values obtained by adding up mean expression levels of the two alleles in the given heterozygous combination (Supplemental Table A.6). An interesting consequence of this finding is that not only can a relative amount of DQA1 mRNA differ from allele to allele, but also the total relative amount of DQA1 mRNA can differ between individuals based on the specific alleles they carry. Indeed, we observed higher than twofold differences in total relative DQA1 mRNA expression in individuals in our cohort (from DQA1/DRA mRNA ratio 0.15 in DQA1*01:01/*05:05 heterozygotes, to more than 0.30 in most DQA1*03/other heterozygotes).

These differences could be even more pronounced in individuals homozygous for the most and the least expressed alleles, but these were not present in our study group (however, we observed up to 4-times higher expression of total relative DQA1 mRNA in DQA1*03/*03 homozygotes compared to DQA1*05:01/*05:01 homozygotes in group of patients with autoimmune disease—unpublished data of our laboratory).

The differences in allele-specific DQA1 expression can have important consequences for immune regulation. It was shown that different amount of either MHC class II molecules on the APCs surface or the presented peptide MHC can lead to preferential development of cells with different effector phenotypes. Multiple experiments were performed, where variation of these parameters was shown to influence Th1–Th2 equilibrium of effector cells [9,10,25–27], and probability of development of regulatory Foxp3+ [28] or inflammatory Th17 cells [27,29].

Thus, we can see function of MHC class II molecule as a combination of both, the spectrum of peptides it presents and the amount of the molecule on the cells surface. In this way, we could speculate that presentation of high concentrations of peptide-MHC complexes with high affinity to TCR leads to preferential induction of pro-inflammatory Th17 cells [29] and suboptimal number of tolerogenic Foxp3+ cells [28]. By far the most expressed DQA1 alleles, DQA1*03, are associated with multiple autoimmune diseases [30–32] and it would be interesting to see whether their high expression level indeed is one of the factors that favor breaking of immune tolerance in their carriers.

4.2. What are the advantages of the use of HLA-DRA gene as an endogenous reference?

Transcription of all HLA class II genes is regulated very tightly and in a coordinated fashion by common class II transactivator, CIITA [1,33]. Without CIITA, there is no class II transcription, and there is strong positive correlation between levels of the CIITA protein in the cell and the level of any of the HLA class II transcripts [33]. In addition, basically all inflammatory stimuli that boost HLA class II expression, act through CIITA [34].

The proportion of HLA class II expressing cells in the whole blood can vary between different individuals and depends on the proportion of APCs among leukocytes and signals that stimulate the HLA class II expression. Furthermore, amount of HLA class II molecules expressed by one cell can differ on a cell-to-cell basis. Thus, referring to classical endogenous control could lead to the finding of different DQA1 allele expression between two samples only due to differences in the amount of HLA class II expressing cells or strength of inflammatory stimuli in the sample. However, as all HLA class II molecules are regulated coordinately, if we use another HLA class II molecule as an endogenous control, we eliminate all these influences. HLA-DRA gene is unique among HLA class II genes because it almost lacks their typical polymorphism. Thus, interindividual variation in its expression should not be affected by genetic variation. As a result, the DRA-normalized expression of DQA1 allele is proportionate purely to the strength of alleles promoter (caused by cis-acting genetic and epigenetic differences).

The use of DRA as an endogenous control gives us one more advantage—we can perform additional expression studies on any other HLA class II gene and relate it to the DRA expression. This is important, as α and β chains form the antigen presenting molecule only together, as a dimer. In a heterozygote for both DQA1 and DQB1 genes, altogether 4 different DQ molecule dimers can appear on the cells surface. By knowing the expression level of the both the DQA1 and both DQB1 alleles, we can estimate the amount all dimers on the cells surface.

Further, even though a linear relationship between the amount of DQA1 and DRA gene mRNA was one of the premises of our

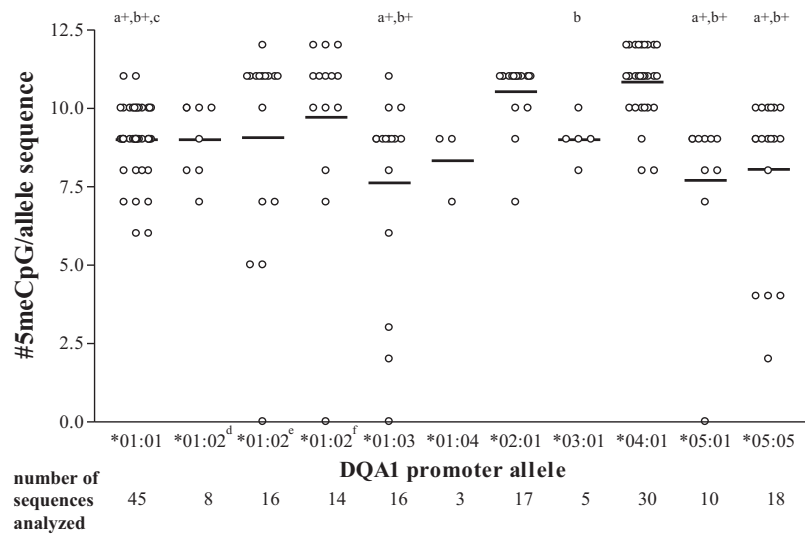


Fig. 3. Overall methylation of DQA1 promoter alleles. According to the polymorphisms present on the sequencing read, sequences were classified into appropriate allelic groups. For each sequence, the total number of methylated CpG sites in the region examined (i.e. overall methylation) was counted. Each point in the graph represents the number of methylated CpGs per 1 sequence. The horizontal line indicates mean methylation level of each allele.

^{a(a+)} Allele promoter methylation significantly, $p < 0.05$ (highly significantly, $p < 0.005$) lower than that of DQA1*02:01 allele.

^{b(b+)} Allele promoter methylation significantly, $p < 0.05$ (highly significantly, $p < 0.005$) lower than that of DQA1*04:01 allele.

^c Allele methylation significantly lower than that of DQA1*01:02 (QAP 1.4 linked) allele.

^d Linked with DQA1 promoter allele QAP 1.2K.

^e Linked with DQA1 promoter allele QAP 1.2L.

^f Linked with DQA1 promoter allele QAP 1.4.

experimental setup, the approximately constant ratio of DQA1 mRNA/DRA mRNA for each DQA1 allele and also for DQA1 total indeed confirms its validity.

4.3. DNA methylation of the polymorphic HLA-DQA1 promoters

Could the observed differences in alleles' expression be caused by variant DNA methylation? One way how DNA methylation can regulate gene's expression is by blocking the access of regulatory proteins to their recognition DNA sequence [35]. In the HLA-DQA1 gene promoter, there are not any CpG dinucleotides present in whole conserved S-X1-X2-Y box region, on which an enhanceosome forms. Thus, regulation by methyl group blocking the transcription factor binding to this area is unlikely. We did not find any significant differences in methylation of specific CpG sites between individual DQA1 promoter alleles, so even if this kind of regulation happens here, the effect on the every allele involved would be same.

The second way of regulating the gene expression by DNA methylation is by the recruitment of chromatin remodeling proteins that induce a repressive chromatin state in the area [35]. In this case, it is plausible that the strength of the effect could depend on the local concentration of methylated cytosines [36].

In the region examined (−641 to −193 relative to first ATG), promoters of DQA1*02:01 and DQA1*04:01 are methylated significantly more than most of the other alleles, and DQA1*05:01 is the least methylated allele. However, the high methylation density of these alleles did not match their average expression levels, and the lower expression of DQA1*05:01 allele was not in accordance with its low methylation density.

It was shown that at least in vitro, proteins recognizing 5-methylcytosine show different preferences to this mark depending on the DNA sequence that surrounds it [37–39]. As a consequence, their affinity towards alleles that have CpGs placed in the different sequence context can differ, even in the case when the alleles are methylated to the same extent. This could be of importance in highly polymorphic class II promoters, whose CpG dinucleotides

vary not only in number, but also in distribution pattern and sequence context, e.g. Methyl-CpG binding protein 2 (MeCP2) prefers CpG sites adjacent to (A/T)_{n≥4} stretch [37], Methyl-CpG-binding domain protein 1 (MBD1) recognizes best methylated Cs in a repeat of several methylated CpGs, and has somewhat higher affinity to CpG in T(G)CCGCA context too [38]. Out of the sequences examined, another methyl-CpG binding protein, Kaiso, had the highest affinity towards those that contain two adjacent CpG dinucleotides [39]. There are no MBD1 binding sites in any of the DQA1 promoter alleles, but 1 Kaiso binding site in promoter of DQA1*02:01 and DQA1*01 group of alleles. It is interesting though that the least expressed alleles, DQA1*05:01 and DQA1*05:05, have most theoretical MeCP2 binding sites (5 compared to 4 in DQA1*03 and 3 in most DQA1*01 alleles). As most of the CpG sites in all alleles are methylated (Fig. 1 and below), these context differences have a chance to manifest. Maybe this could be one of the factors that add up and lead to low expression of the DQA1*05 alleles.

In all alleles we could observe the common pattern where most of the promoter CpG sites were methylated and as we approached the transcription start site, the methylation decreased to almost zero. According to the criteria of Weber et al. [36], all DQA1 allele promoters belong among low-CpG promoters, the class of promoters with low content of CpG that tend to be highly methylated whether they are expressed or not, thus their high methylation should not be an obstacle to alleles' expression. However Weber's analysis simplified the gene expression level to yes/no sense, and their findings do not exclude fine regulation of gene expression by DNA methylation even in this promoter class. Majumder et al. showed that DNA hypermethylation of the promoter region of the DQA1 and DQB1 genes in cancer cells is able to suppress the transcription of the gene even in the presence of CIITA and all other factors necessary for class II genes' expression [40,41]. For DQA1 gene, the region in question largely overlapped with the region that was unmethylated in our samples. Altogether these data hint that in spite of the relatively low content of CpG dinucleotides in DQA1 gene promoter, high level of their methylation in an area very proximal to the transcription start site is able to decrease gene's

expression and maybe this could be the site of the major regulation. We observed that this area tends to be methylation free.

To conclude, we suppose that the above mentioned interallelic differences in context and positioning of CpG dinucleotides, and not less importantly also the polymorphisms on the level of DNA sequence, could be the major forces driving expression differences of individual DQA1 gene alleles.

4.4. Conclusions

This study describes DNA methylation pattern of promoter region of HLA class II DQA1 gene alleles and proves interallelic DNA methylation difference. It also contributes to the elucidation of mRNA expression hierarchy of DQA1 alleles. However, the correlation between these two characteristics has not been revealed.

The advantage of our study is the use of the HLA-DRA gene as an endogenous control. Even though the RNA for analyses was isolated from the whole peripheral blood, normalizing the DQA1 expression against the DRA expression level allowed us to study levels of HLA-DQA1 mRNA expression specifically in HLA class II expressing cells of the blood. Furthermore, this approach opens up the possibility to compare results obtained by independent expression studies on any other HLA class II gene.

The shortcoming of our study could be that the DNA methylation was studied in unsorted whole blood leukocytes, while the expression of DQA1 alleles was inspected in HLA class II expressing cells.

As a next step, due to important role of HLA class II genes in autoimmune diseases, we would like to determine DNA methylation and expression profile of other HLA class II genes.

Conflict of interest

The authors have no conflicts of interest in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imlet.2015.08.006>.

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DNA methylation and mRNA expression of HLA-DQA1 alleles in type 1 diabetes mellitus

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Summary

Type 1 diabetes (T1D) belongs among polygenic multifactorial autoimmune diseases. The highest risk is associated with human leucocyte antigen (HLA) class II genes, including HLA-DQA1 gene. Our aim was to investigate DNA methylation of HLA-DQA1 promoter alleles (QAP) and correlate methylation status with individual HLA-DQA1 allele expression of patients with T1D and healthy controls. DNA methylation is one of the epigenetic modifications that regulate gene expression and is known to be shaped by the environment. Sixty one patients with T1D and 39 healthy controls were involved in this study. Isolated DNA was treated with sodium bisulphite and HLA-DQA1 promoter sequence was amplified using nested PCR. After sequencing, DNA methylation of HLA-DQA1 promoter alleles was analysed. Individual mRNA HLA-DQA1 relative allele expression was assessed using two different endogenous controls (PPIA, DRA). We have found statistically significant differences in HLA-DQA1 allele 02:01 expression (PPIA normalization, $P_{\text{corr}} = 0.041$; DRA normalization, $P_{\text{corr}} = 0.052$) between healthy controls and patients with T1D. The complete methylation profile of the HLA-DQA1 promoter was gained with the most methylated allele DQA1*02:01 and the least methylated DQA1*05:01 in both studied groups. Methylation profile observed in patients with T1D and healthy controls was similar, and no correlation between HLA-DQA1 allele expression and DNA methylation was found. Although we have not proved significant methylation differences between the two groups, detailed DNA methylation status and its correlation with expression of each HLA-DQA1 allele in patients with T1D have been described for the first time.

Keywords: DNA methylation; HLA class II genes; HLA-DQA1 promoter (QAP); mRNA expression; type 1 diabetes mellitus.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease that leads to the selective destruction of pancreatic β -cells and to lifelong dependence on exogenous insulin. Its worldwide incidence is increasing at a rate of nearly 3% per year.¹ Nearly 40 genes and gene complexes contribute to T1D risk, including protein tyrosine phosphatase, non-receptor type 22, cytotoxic T-lymphocyte-associated pro-

tein 4 (CTLA-4) and insulin genes.² The strongest genetic risk (about 50%) is associated with HLA class II complex (particularly with its DQ and DR regions) located on the short arm of chromosome 6. Heterozygotes for DRB1*04-DQA1*03:01-HLA-DQB1*03:02 and DRB1*03-DQA1*05:01-DQB1*02:01 haplotypes carry the highest risk for T1D development in the Caucasian population. In contrast, the allele DQB1*06:02 (part of DRB1*15-DQA1*02:01-DQB1*06:02 haplotype) is negatively associ-

Abbreviations: CD14⁺, cluster of differentiation 14; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IPTG, isopropyl- β -D-thiogalactopyranoside; HC, healthy controls; HLA, human leucocyte antigen; PPIA, peptidylprolyl isomerase A; QAP, HLA-DQA1 promoter; T1D, type 1 diabetes mellitus; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galacto-pyranoside

ated with the disease.³ The presence of a single copy of this allele is sufficient to protect from T1D. Although many studies have confirmed the association between T1D and HLA-DQ and HLA-DR molecules, their role in T1D aetiopathogenesis is not fully understood. One possibility is that HLA-DQ risk alleles bind autoantigens with low affinity and that leads to the aberrant selection of T-cell repertoire in thymus or periphery.⁴ Another possibility is that autoimmunity could be associated with low expression of HLA molecules. This would result in a general decrease in the number of HLA class II molecules on the cell surface and hence to less efficient presentation of autoantigens to protective regulatory T cells.⁵

Not only genetics but also an environment can modulate T1D risk in genetically susceptible individuals. Concordance values in monozygotic twins, ranging from 25 to 60%, clearly demonstrate the relation between T1D manifestation and environmental factors.^{6,7} Furthermore, in recent years, more people with low risk or even protective HLA genotypes are becoming prone to developing T1D.^{8,9} This could be caused by gene–environment interactions leading to aberrant DNA methylation of genes regulating T1D susceptibility, including HLA class II locus, and causing different expression of HLA class II alleles. Recent studies showed a distinct DNA methylation profile in T1D risk genetic regions, like in the insulin promoter or HLA class II region.^{10–12} However, it remains to be elucidated how this methylation difference influences T1D susceptibility and whether these changes correlate with changes in the expression of T1D risk genes. So far, this was only observed in the insulin gene, where hypermethylation of one CpG dinucleotide within the promoter region correlated *in vitro* with low expression of insulin.¹³

In this study we focused on DNA methylation of HLA-DQA1 gene promoter (QAP). It has been shown that DQA1 promoter alleles have different strengths and can influence the expression level of the HLA-DQA1 gene.^{14–16} We hypothesized that HLA class II expression could be regulated not only by genetic polymorphisms, but also by epigenetic modifications including DNA methylation within the promoter region. To confirm this hypothesis we analysed the DNA methylation status of individual HLA-DQA1 promoter alleles. We tried to understand the relationship between allele's methylation and its mRNA expression level. Finally we compared these characteristics between patients with T1D and healthy controls.

Material and methods

Subject

The participants in this study consisted of 61 patients with T1D (45 men and 16 women; median age of 32.5 years; average age 39.8 years) and 39 healthy

individuals (17 men and 22 women; median age of 34.5 years; average age of 39.1 years) of Caucasian origin and from the same ethnic background. The study was approved by the Ethical Committee of the Third Faculty of Medicine of Charles University in Prague and the written informed consent was obtained from each subject. All patients with T1D were diagnosed at the University Hospital Kralovske Vinohrady, Prague, Czech Republic. The diagnosis of diabetes was made according to published criteria.¹⁷ Autoimmune origin was confirmed by very low levels of C-peptide and/or positive serum autoantibodies against either insulin, or glutamic acid decarboxylase, or islet antigen islet antigen-2.

Age-matching controls were chosen according to their HLA haplotypes to correspond with HLA haplotypes of patients with T1D. The controls were not tested for the presence of specific autoantibodies, however, only healthy individuals (self-reported) with neither diabetes and diabetes-associated symptoms nor any other autoimmune disease were included in the study. Characteristics of the subject population are shown in Table 1. Mann–Whitney non-parametric test did not prove any statistically significant differences between the ages of both groups ($\alpha = 0.05$).

DNA isolation and bisulphite sequencing

From each subject, 20 ml of whole peripheral blood was collected into tubes with 3% EDTA. Genomic DNA from whole blood (patients with T1D and healthy controls) and CD14⁺ monocytes (only patients with T1D) were extracted by the salting out method and treated with sodium bisulphite using an Epitect Bisulfite kit (Qiagen, Hilden, Germany). CD14⁺ monocytes were isolated using Dynabeads CD14[®] (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.¹⁸ HLA-DQA1 gene promoter region was amplified by nested PCR in three separate reactions that were mixed together after PCR; target fragments were separated by 1% TAE agarose gel electrophoresis and purified by a MinElute Gel Extraction kit (Qiagen). For amplification, the following sets of primers were used:

F1 5'-GGT TGT AAG TTA GAA TAT TTT GAA GGA TG-3' and R1 5'-CAA ACC AAA CCC TAC CAA ATC A-3' for the first PCR; and F2 5'-AGG TTG TTT AGA AAT GTT TAT TTT TGG-3' and R2 5'-AAA ATC CCC TAT AAT AAC ATC TCA ATT AC-3' for the second reaction. PCR conditions were reported in Zajacova *et al.*¹⁶ The 545-bp long amplicon was inserted into the pGEM-T easy vector (Promega, Madison, WI) and cloned into the *Escherichia coli* DH5 α strain. Positive colonies were selected on agar plates containing ampicillin (100 mg/ml), X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galacto-pyranoside, 3%), IPTG (isopropyl- β -D-thiogalactopyranoside, 100 mM) and confirmed by colony PCR

Table 1. Characteristics of study subjects

Group	Patients with type 1 diabetes mellitus ¹ (<i>n</i> = 61)	Healthy controls ² (<i>n</i> = 39)
Number of males/females	45/16	17/22
Age in years range (median and mean)	21–70 (32.5 and 39.8)	20–77 (34.5 and 39.1)
Men's age in years range (median and mean; SD)	21–70 (36.0 and 41.5; 14.8)	20–77 (40.0 and 40.1; 15.5)
Women's age in years range (median and mean; SD)	21–68 (31.0 and 33.9; 19.1)	25–75 (28.0 and 38.3; 17.8)
Duration of T1D in years range (median and mean)	5–42 (14.0 and 15.2)	–

¹Individuals with overt hyperglycaemia and at least one of specific autoantibodies.

²Individuals with no overt disease symptoms and with no history of any autoimmune diseases.

using universal SP6 and T7 primers. At least six different positive colonies from each patient were sequenced by Sanger method with fluorescence-labelled nucleotides in MacroGen (Seoul, Korea). Obtained sequences were aligned according to the reference sequence of HLA-DQA1 (ENSG00000196735) in BIOEDIT software, version 7.0.9.0 (Carlsbad, CA, USA). Only sequences where bisulphite treatment was at least 95% successful were taken into consideration. Damaged or recombined sequences were removed from analysis.

HLA-DQA1, DRB1 and DQB1 genotyping

For genotyping, HLA Olerup SSP™ typing kits (Olerup AB, Stockholm, Sweden) were used. First, HLA-DQ and DR low-resolution and HLA-DQA1 genotyping was done. Second, HLA-DQB1 high-resolution typing using allele-specific Olerup SSP™ HLA-DQB1 typing kits was performed according to the manufacturer's instructions. PCR products were identified by 2% agarose gel electrophoresis and evaluated according to manufacturer's instructions.

RNA isolation and mRNA expression

Total RNA was isolated from whole blood using Gen Elute™ Miniprep Kit (Sigma Aldrich, St Louis, MO). The cDNA was prepared by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 200 ng input RNA. Sequences of primers and probes used in real-time PCR were reported previously.¹⁶ The 'DQA1 total' assay was used to quantify total DQA1 mRNA irrespective of alleles present, the 'DRA' assay was used as an endogenous control and the 'DQA1 intron' assay amplifying intronic segment of DQA1 gene was used to determine genomic DNA contamination of our samples. All measurements were done using 7500 Fast Real time PCR system (Applied Biosystems) with fluorescent Taqman™ probes and

primers. All samples were measured in triplicate and contained 200 nM of probe, 300 nM of each primer and 1× Gene Expression Master Mix (Applied Biosystems) using the following cycling conditions: 50° for min and 95° for 10 min, followed by 40 cycles of 95° for 15 seconds and 60° for 1 min. For each assay, an amplification efficiency was determined and ranged between 95% and 102%.

Statistical analysis

GRAPHPAD PRISM 5.0.4 software was used for statistical assessments (GraphPad Software, La Jolla, CA, USA). Differences in overall DNA methylation of DQA1 promoter alleles were evaluated by a two-tailed non-parametric Mann–Whitney test with significance level of $\alpha = 0.05$. Statistically significant results were corrected using Bonferroni correction (P multiplied by number of promoter alleles tested, $P_{\text{corr}} = P_{\text{uncorr}} * 10$). Significant differences between allele frequencies of patients with T1D and healthy controls were determined using chi-square test ($\alpha = 0.05$) with Bonferroni correction ($P_{\text{corr}} = P_{\text{uncorr}} * 10$). Differences between individual methylation positions were calculated using Fisher's exact test with $\alpha = 0.05$ with Bonferroni correction ($P_{\text{corr}} = P_{\text{uncorr}} * 10$). Methylation differences between whole blood leucocytes and monocytes of patients with T1D were tested using a non-parametric Kruskal–Wallis test with Dunn's post-test at the level of significance $\alpha = 0.1$.

Relative expression of DQA1 alleles was determined with reference to the amount of HLA-DRA mRNA. Only samples in which the ratio of the sum of both alleles mRNA to the amount of total DQA1 mRNA was in the interval between 0.85 and 1.15 were included in the analyses. Relative expression between all possible pairs of alleles was compared by a two-tailed Mann–Whitney test with 95% CI. All P -values were subjected to Bonferroni correction for the number of alleles compared ($P_{\text{corr}} = P_{\text{uncorr}} * 10$).

Results

Identification of individual DQA1 promoters (QAP alleles)

DQA1 promoter alleles were identified according to the known sequences found in the IMGT/HLA database (<http://www.ebi.ac.uk/ipd/imgt/hla/>). In total, 10 different promoter alleles in patients with T1D and 11 promoter alleles in healthy controls (HC) were detected. Most of the promoter alleles were in linkage disequilibrium with one respective DQA1 allele, except DQA1*01:02 allele, which was associated with two different QAP alleles 1.2K, and 1.4. While QAP 1.2K was associated with DR*16-DQA1*01:02-DQB1*05:02, QAP 1.4 was associated with DR*13-DQA1*01:02-DQB1*06:04. The association between DQA1 alleles and promoter alleles (QAP) is depicted in the Supplementary material (Table S1). Two patients were detected with a protective haplotype DRB1*13-DQA1*01:03-DQB1*06:03. These patients had manifested disease at 40 and 41 years of age, respectively.

Normalization against two different endogenous controls

As an endogenous control to normalize mRNA expression of HLA-DQA1 alleles we used another HLA class II gene, the non-polymorphic HLA-DRA, which is located in the linkage disequilibrium region together with the DRB1, DQB1 and DQA1 genes. Transcription of all these genes is regulated by the same transcription factors (RFX, X2BP, NF-Y and the class II transactivator CIITA). Hence, the normalization against the DRA gene allows us to correct for the increase of DQA1 expression caused not by differences in promoter strength, but by inflammation. We hypothesized that expression of HLA class II genes including the DQA1 gene could be changed by the health status of the patient, so we explored this hypothesis by normalizing the expression data against another endogenous control, peptidylprolyl isomerase A (PPIA). This approach allowed us to see the differences in individual DQA1 allele expression between healthy controls and patients with T1D that were caused by altered inflammatory state or by factors other than promoter strength in patients with autoimmune disease.

Relative expression of individual DQA1 alleles HLA-DRA normalized

In total, mRNA levels of DQA1 alleles in 43 patients with T1D (36 heterozygotes and seven homozygotes) and 39 healthy controls (34 heterozygotes and five homozygotes) were analysed in our study. DQA1*03 allele was significantly more expressed in patients with T1D than all other analysed alleles (DQA1*01:01; 01:02K; 02:01; 03:01 and 05:01). In healthy controls, the DQA1*03 allele was significantly more

expressed than 01:01; 01:02L; 01:03; 01:04; 02:01; 03:01; 05:01 and 05:05. Furthermore DQA1*0103 allele was significantly more expressed than 01:01, 02:01, 05:01, 05:05. (Fig. 1).

DQA1 alleles for which fewer than three expression values were obtained (T1D: 01:02M, 01:03, 01:05, 04:01, 05:05; HC: 0102K, 0102M, 04:01) were not included in the statistical analysis. Our data indicate that promoters of DQA1*01:03 and DQA1*01:02M alleles may be stronger than DQA1*01:01, DQA1*01:02L (Fig. 1). Statistical analysis revealed no significant differences between DRA-normalized DQA1 allele expression in patients with T1D and healthy controls. However, expression of DQA1*02:01 in healthy controls compared with patients with T1D ($P_{\text{corr}} = 0.052$) was at the edge of significance. All results are summarized in Fig. 1.

Relative expression of individual DQA1 alleles normalized against PPIA

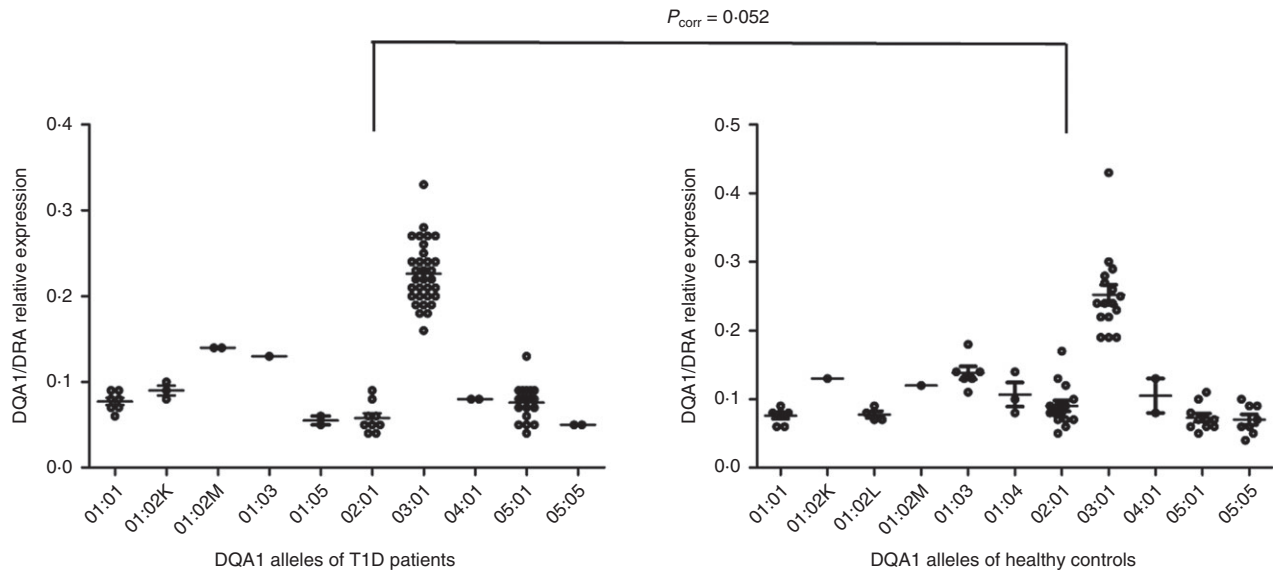
Normalization against PPIA revealed significantly higher expression of the DQA1*02:01 allele in healthy controls compared patients with T1D ($P_{\text{corr}} = 0.041$). Higher expression of DQA1*05:01 allele in patients with T1D compared with healthy controls lost significance after correction. All other observations from PPIA normalization were similar to those from HLA-DRA normalization. In support of our hypothesis for using two endogenous controls, the expression data from normalization against PPIA (Fig. 2) were much more variable than the data from normalization against HLA-DRA gene, especially for DQA1*03 and DQA1*01:01 alleles.

Unequal expression of DQA1*02:01 allele in DQA1 02:01/03 heterozygotes

Expression of DQA1*02:01 was higher in healthy controls, so we investigated if there was a haplotype combination with the most marked differences in DQA1*02:01 expression. We calculated mean allele expression ratio between different haplotypes of patients with T1D and healthy controls (see Supplementary material, Table S2). We found that the mean allele expression ratio of DQA1 03/02:01 heterozygotes was almost two times higher (not significant) in patients with T1D (4.86) than in healthy controls (2.96). Relative expression of DQA1*02:01 and DQA1*03 alleles of T1D and healthy individuals is illustrated in the Supplementary material (Fig. S1), mean allele expression ratios of all different haplotype combinations are summarized in Table S2 (see Supplementary material).

Methylation variances between whole blood leucocytes and monocytes of patients with T1D

Using the bisulphite sequencing method, we determined methylation status of 9–12 CpGs per sequence depending



Explanation:

01:02K = DQA1*01:02 allele in linkage with QAP 1.2K allele

01:02L = DQA1*01:02 allele in linkage with QAP 1.2L allele

01:02M = DQA1*01:02 allele in linkage with QAP 1.4 allele

Figure 1. Relative expression of individual DQA1 alleles normalized against HLA-DRA. Each point in the graph represents normalized value for one individual and one allele. The horizontal line indicates mean relative expression of the allele. Mean values with standard error of the mean are indicated. In total 43 patients with type 1 diabetes mellitus (T1D) and 39 healthy controls were analysed in this study. Difference in expression of DQA1*02:01 in healthy controls and patients with T1D was at the edge of significance ($P_{\text{corr}} = 0.052$). DQA1*03 was significantly more expressed than all other alleles as in patients with T1D so in healthy controls. DQA1*05:05 has the lowest expression in both studied groups. Statistical significance was tested by Mann–Whitney test ($\alpha = 0.05$) followed by Bonferroni correction ($P_{\text{corr}} = P_{\text{uncorr}} * 10$).

on QAP allele identity. Methylation profile of CD14⁺ monocytes and whole blood leucocytes of patients with T1D was obtained. We tested for significant differences between both cell populations including total QAP methylation and specific QAP methylation of individual CpGs. For this purpose, we compared all eight DQA1 alleles between whole blood leucocytes and monocytes using non-parametric Kruskal–Wallis test with Dunn's post-test at the level of significance $\alpha = 0.1$. This test revealed no statistically significant differences between total methylation of individual DQA1 alleles of whole blood leucocytes and monocytes (see Supplementary material, Fig. S2). This approach allowed us to combine the sequences from monocytes and whole blood leucocytes of patients with T1D into one group and compare them with whole blood leucocyte sequences from healthy controls.

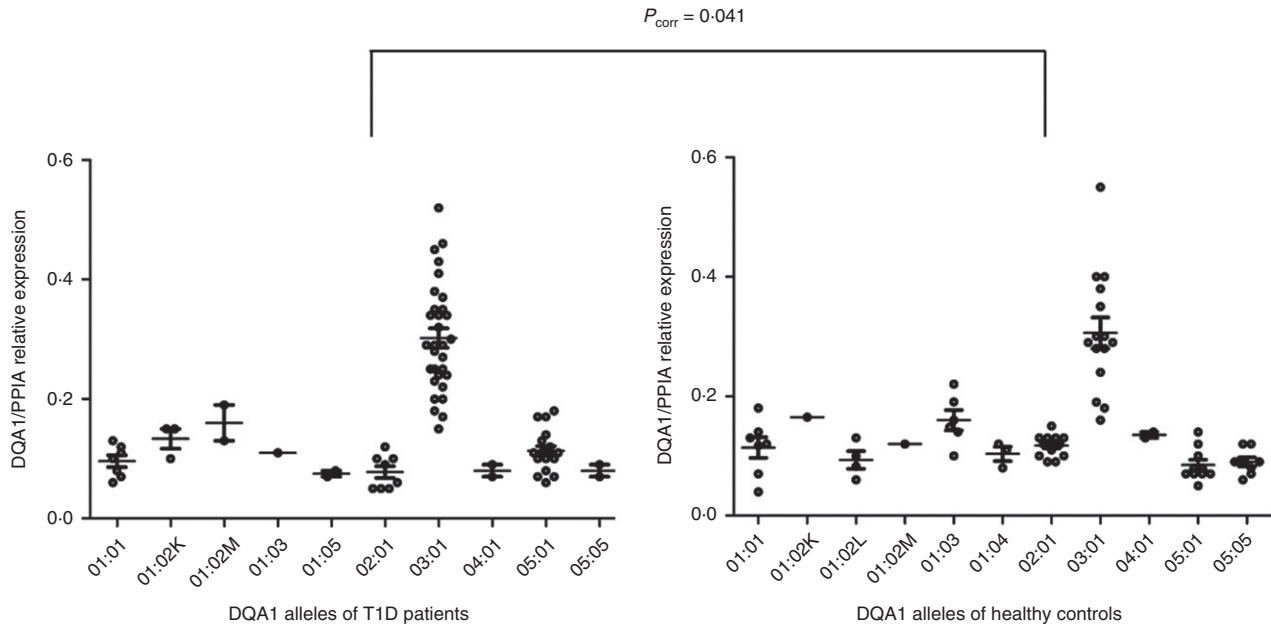
When comparing the specific QAP methylation of individual CpGs, we found one statistically significant difference between whole blood leucocytes and monocytes of patients with T1D in DQA1*01:02M promoter at the position -311 ($P_{\text{corr}} = 0.020$). Monocyte DQA1*01:02M sequences were collected from only one patient, who had this position (-311) completely unmethylated. This was not observed in whole blood leucocytes where sequences came from three different people and where the position

-311 was more methylated (70%). For this reason we decided to exclude 01:02M monocyte sequences from further analysis and use only sequences from whole blood leucocytes.

HLA-DQA1 promoter methylation of T1D patients and healthy controls

In both studied groups, the most methylated promoter allele was DQA1*04:01 (part of DRB1*08 haplotype) and DQA1*02:01 (DRB1*07 haplotype). In contrast, the least methylated promoter was DQA1*05:01 (DRB1*03 haplotype) allele. Results are summarized in Fig. 3.

When we analysed the specific methylation of individual promoter CpGs in two study groups, we revealed that while CpG dinucleotides from region -641 to -374 are almost completely methylated, as we get closer to the transcription initiation site, DNA methylation level decreases to almost no methylation at position -193 (Fig. 4a,b). Moreover, closer to the transcription initiation site, a more distinct methylation pattern is observed between patients with T1D and healthy controls. The most differences in individual CpG dinucleotide methylation between both groups were found at the position -311 . Although the promoter of DQA1*02:01 allele was



Explanation:

01:02K = DQA1*01:02 allele in linkage with QAP 1.2K allele

01:02L = DQA1*01:02 allele in linkage with QAP 1.2L allele

01:02M = DQA1*01:02 allele in linkage with QAP 1.4 allele

Figure 2. Relative expression of individual DQA1 alleles normalized against peptidylprolyl isomerase A (PPIA). Each point in the graph represents normalized value for one individual and one allele. The horizontal line indicates mean relative expression of the allele. Mean values with standard error of the mean are indicated. DQA1*02:01 was significantly more expressed in healthy controls than in patients with type 1 diabetes mellitus (T1D) ($P_{\text{corr}} = 0.041$). HLA-DQA1*05:01 expression was elevated in patients with T1D compared with healthy controls, but significance was lost after correction ($P_{\text{corr}} = 0.369$). Normalization against PPIA was performed to analyse differences in individual DQA1 allele expression between healthy controls and patients with T1D caused by altered inflammatory state or by other factors different than promoter strength in patients with autoimmune disease.

almost completely methylated (78% HC, 93% T1D) at this position, methylation of other promoter alleles ranged between 33% and 63% (33–63% HC, 37–55% T1D). However, statistical analysis showed no significance regarding the DQA1 promoter methylation status between patients with T1D and healthy controls. We also compared DNA methylation of T1D risk and protective haplotypes of patients with T1D with healthy controls that carry the same T1D risk and protective haplotypes. No methylation differences were found either for T1D protective DR*13-DQA1*01:03-DQB1*06:03 or for T1D-risk haplotypes DR*04-DQA1*03-DQB1*03:02, DR*03-DQA1*05:01-DQB1*02:01.

Discussion

HLA-DQA1 expression is not well mapped and only a few studies have characterized its expression in patients with T1D and healthy controls. A study by Maffei *et al.*, confirmed by Donner *et al.*, revealed higher expression of DQA1*03 in DQA1*03/DQA1*05:01 healthy heterozygotes.^{19,20} These studies were followed by study from Fernandez *et al.*, where in contrast to other works the

highest DQA1 expression was found for DQA1*04 allele and the lowest for DQA1*02, 03 and 05 alleles.²¹ So far the most extensive work was published by Britten *et al.*, where not only individual allele expression was performed but also promoter activity assays were carried out.¹⁵ In general, we found the highest expression for DQA1*03 allele and the lowest for DQA1*05:05 allele in both groups, which is in accordance with the work of Britten and Donner. DQA1*03 was significantly more expressed than the other DQA1 alleles in both studied groups. Alleles where expression values for only two samples were obtained could not be statistically analysed. The most variable expression was found for DQA1*01 allele, where DQA1 expression was dependent on the specific DQA1 promoter (QAP). We did not observe HLA-DQA1 expression differences in T1D risk or protective haplotypes between patients with T1D and healthy controls. However, we found a statistically significant increase in expression of HLA-DQA1*02:01 allele in healthy controls. To our knowledge, we are the first to report this observation. This allele is in linkage disequilibrium (part of haplotype) with HLA-DRB1*07 and HLA-DQB1*02:02 and is neither a risk nor protective for T1D.

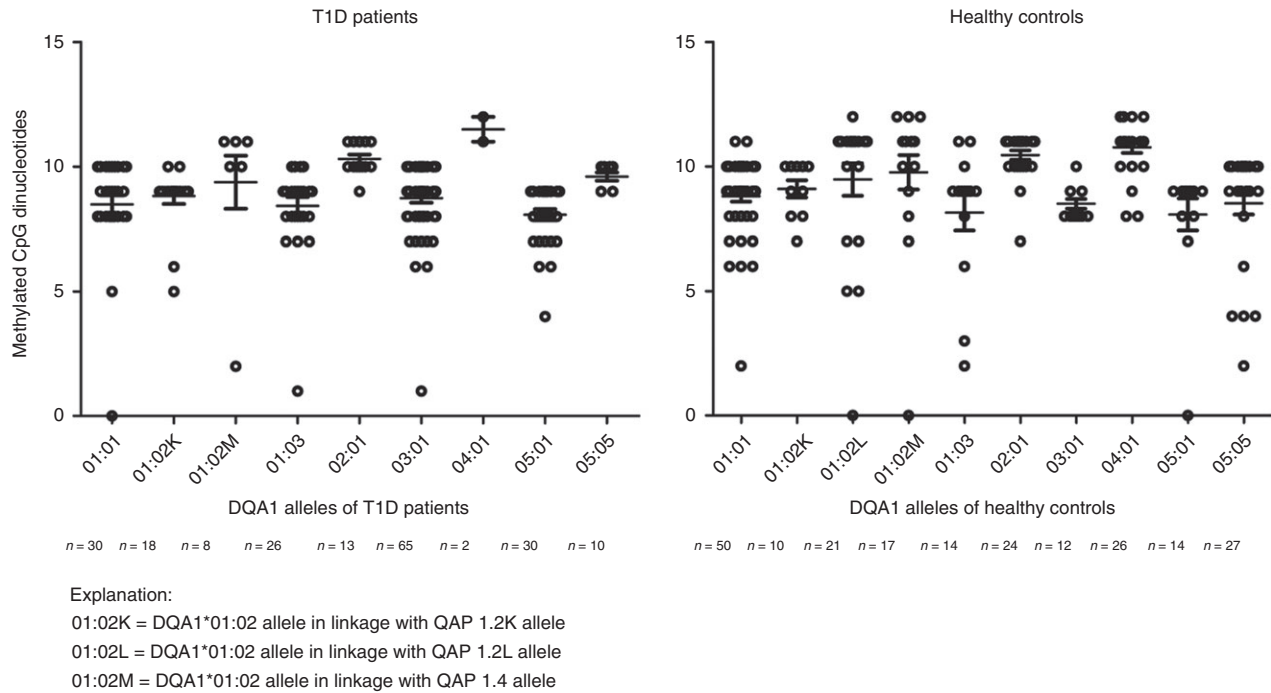


Figure 3. Total methylation profile of DQA1 promoter alleles in patients with type 1 diabetes mellitus (T1D) and healthy controls. Each point in the graph represents number of methylated CpG dinucleotides from single sequence of monocyte or whole blood leucocyte. Mean values with standard error of mean are indicated. Number of methylation positions was dependent on QAP allele identity (9–12 CpGs per sequence). The most methylated allele was DQA1*04:01 allele (12 CpGs per sequence) and DQA1*02:01 allele (11 CpGs per sequence) in both studied groups. The least methylated allele was 05:01 (nine CpGs per sequence). Statistical analysis showed no statistical difference in total methylation of DQA1 promoter alleles between patients with T1D and healthy controls. Statistical significance was tested by Mann–Whitney test ($\alpha = 0.05$) followed by Bonferroni correction ($P_{\text{corr}} = P_{\text{uncorr}} * 10$).

HLA class II expression is mainly regulated at the transcription level by the SXY module, which is localized –150 to –300 bp before the transcription initiation site. All MHC class II genes include this motif and this is where the enhanceosome forms. Moreover, additional SXY modules are scattered across the MHC class II locus at distal positions and can function as enhancers.^{22,23} Therefore it is possible that increased DQA1 expression could be related to *cis* and *trans* acting elements upstream of the promoter. Epigenetic mechanisms can also be involved in this type of gene expression regulation, for example, Mio *et al.* demonstrated that the upstream region of HLA-DRB1 and HLA-DQB1 genes has an increased response to interferon- γ and tumour necrosis factor- α that is accompanied by histone H3 lysine 9 acetylation.²⁴

The link between decreased expression of DQA1*02:01 in patients with T1D and T1D pathogenesis is not clear. Possible explanation is that decreased DQA1 expression can influence the spectrum of T-cell-produced cytokines and possibly the shape of immune response. It was shown that the amount of the particular HLA–peptide complex on a cell’s surface influences the amount of the cytokines interleukin-4 and interferon- γ produced by the triggered T cells and consequently the T helper type 1/type 2 response balance.²⁵ It is interesting that by far the most

expressed allele DQA1*03 is part of the DRB1*04-DQA1*03-DQB1*03 haplotype, which is highly predisposing to many autoimmune diseases (like T1D, dominated by a T helper type 1 response). To explore this hypothesis, mRNA expression analysis of the DQA1 partner molecule DQB1 should be performed, and also the total amount of DQ $\alpha\beta$ dimer on the cells’ surfaces should be assessed, because the total amount of the DQ $\alpha\beta$ –peptide trimers on the cell surface does not depend only on the availability of subunits α , β and peptide, but also on the stability of the resulting trimer. As we have performed whole blood leucocyte expression analysis, expression in more immunologically T1D-relevant cell populations (monocytes and B lymphocytes) should be assessed. Another possibility is that autoimmunity could be related to low expression of HLA class II alleles, which results in less efficient presentation of autoantigen to protective regulatory T cells, as published by Swanberg *et al.*⁵ Swanberg *et al.* also described a polymorphism (168A→G) in the MHC2TA gene that is associated with reduced MHC class II expression.⁵ Interestingly, in our study, higher DQA1*02:01 expression was mainly noticeable in 02:01/03 healthy heterozygotes where the ratios between these allele combinations was almost two times lower in healthy controls in comparison with

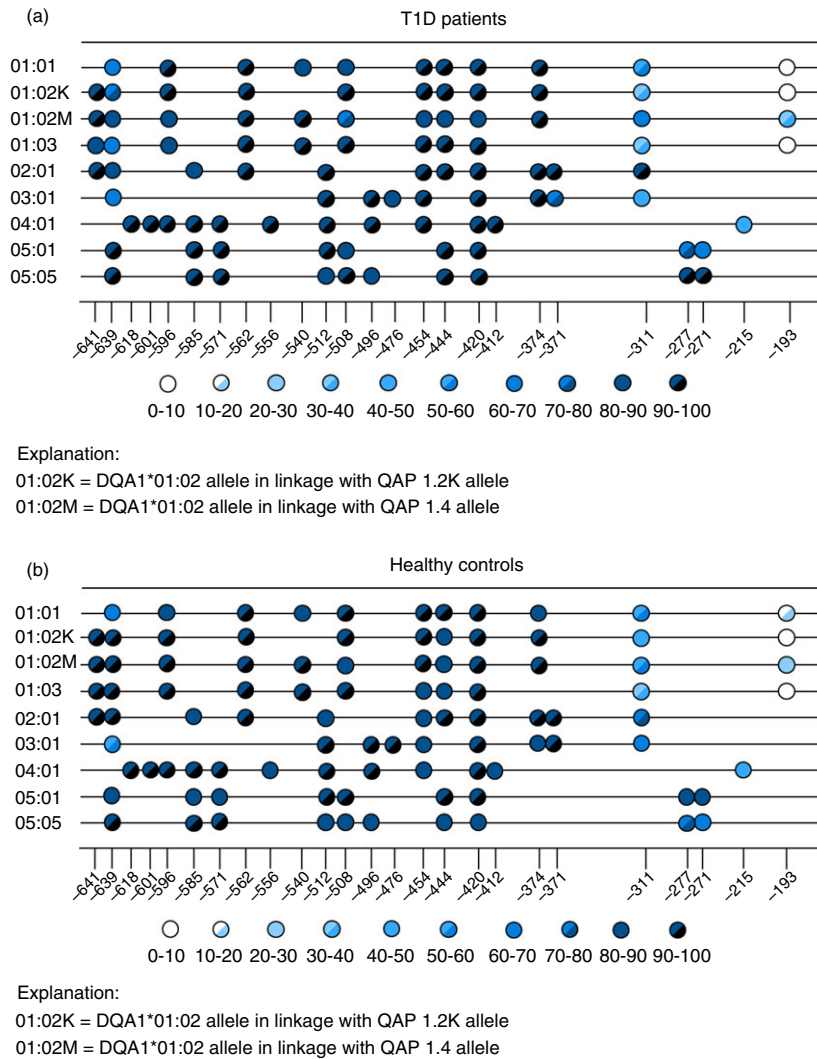


Figure 4. (a) Specific methylation profile of DQA1 promoter CpG sites in patients with type 1 diabetes mellitus (T1D). The matrix represents an amplicon that contains 9–12 CpG sites (number depends on promoter allele identity) obtained for 545 bp region overlapping promoter of the DQA1 gene. Each colour-coded circle within the matrix corresponds to one CpG site and its colour indicates the percentage of sequences that have the cytosine at the given site methylated. Individual methylation positions under figure marks relative position of CpG dinucleotide before initiation transcription site. CpG dinucleotide at the position -311 , -277 , -211 was the most variable in methylation between both studied groups. DQA1*02:01 allele at the position -311 was almost completely methylated (93%). Methylation of other alleles ranged between 37 and 55%. (b) Specific methylation profile of DQA1 promoter CpG sites in healthy controls. The matrix represents an amplicon that contains 9–12 CpG sites (number depends on promoter allele identity) obtained for a 545-bp region overlapping promoter of the DQA1 gene. Each colour-coded circle within the matrix corresponds to one CpG site and its colour indicates the percentage of sequences that have the cytosine at the given site methylated. Individual methylation positions under figure marks position of CpG dinucleotide before initiation transcription site. CpG dinucleotide at the position -311 , -277 , -211 was the most variable in methylation between both studied groups. DQA1*02:01 allele at the position -311 was methylated in 78%, whereas methylation of other promoter alleles ranged between 33 and 63%.

patients with T1D. We are not the first to report different 02:01/03 ratios between healthy controls and patients with T1D. A similar trend was already described in Donner's work, but likewise in our work, allele ratios were not significant due to the small amount of data.²⁰ DQA1*04:01 allele tends to be more expressed in patients with T1D, but only when normalizing against PPIA. As explained earlier, this difference can be caused by inflammatory processes present in patients with T1D but not in healthy

controls, and therefore not visible in DRA normalization. This tendency to higher allele expression in patients with T1D was visible also for other DQA1 alleles, but none of the differences was significant.

We also analysed two heterozygous diabetic patients with DRB1*13-DQA1*01:03-DQB1*06:03 and DRB1*04-DQA1*03:01-DQB1*03:02 haplotypes. It is known that the DRB1*13-DQA1*01:03-DQB1*06:03 haplotype is protective for T1D development, even though some stud-

ies state that the protection is lost when in combination with the DRB1*04-DQA1*03:01-DQB1*03:02 haplotype.²⁶ Despite this fact, both patients have developed autoimmunity relatively late in life, at around 40 years of age. Expression analysis of DQA1*01:03 and DQA1*03:01 did not show aberrant expression compared with the same haplotypes of the controls.

T1D monozygotic concordance values (25–65%) point in favour of an environmental contribution to T1D, which can be expressed through distinct epigenetic profile. Since we have found higher DQA1*02:01 expression in healthy controls, we explored a hypothesis that lower 02:01 expression in patients with T1D could be related to altered DNA methylation within HLA-DQA1 promoter and might increase predisposition to disease, particularly in DQA1*02:01/03 heterozygotes. We have not found simple correlation for HLA-DQA1 expression and DNA methylation. The low expressed alleles 05:05 and 02:01 tend to be highly methylated, but the ‘expression level inversely correlated with methylation density’ principle could not be applied to the most expressed DQA1*03 allele, which has average methylation level. Comparison of total DNA methylation status of HLA-DQA1 promoter alleles between healthy controls and patients with T1D did not reveal any significant differences. Since we used a very fine technique for analysing DNA methylation status, we were able to analyse individual methylation positions in detail. The more we approached the transcription initiation site, the lower the methylation level was. Similar methylation pattern (at individual CpG methylation positions) was detected in patients with T1D and healthy controls, with the most variable methylation site being located at positions –311 (in DQA1*01:01, 01:02K, 01:02M, 01:03, 02:01, 03:01 alleles) and –277, –271 (in DQA1*05:01, 05:05) before transcription initiation site. Kuroda *et al.* showed that insulin expression can be influenced by methylation of one particular CpG site.¹³ If this is the case, then DNA methylation mark could regulate binding of transcription factors to their target sequences. CpG methylation at the position –311, –277, –271 seems to be the best candidate for this type of regulation.

In recent years, increasing attention is focused on epigenetic modifications in T1D. Stefan *et al.* described differences in DNA methylation profiles between T1D concordant and discordant monozygotic twins in HLA and insulin genes.¹² Moreover, a distinct DNA methylation profile was detected in study by Fradin *et al.*, where patients with T1D showed significantly decreased methylation at three CpG positions in the promoter region of the insulin gene.¹¹ Another study by Rakyan *et al.* analysed DNA methylation of CD14⁺ monocytes and found 132 T1D methylation variable positions associated with various genes, including HLA-DQB1 and RFXAB, an HLA class II regulating element.¹⁰ Regarding this information, Majumder *et al.* showed

that two important transcription factors that help to form enhanceosome, RFX and CIITA, did not bind to the hypermethylated promoter proximal regions of HLA-DQA1 and DQB1 genes. Inhibition of methyltransferases restored binding of both factors and led to high HLA-DQA1 and DQB1 expression.^{27,28} These results suggest an importance of the promoter proximal regions of these genes and their methylation status. However, this observation was made in the cell lines derived from acute lymphocytic leukaemia, where different types of regulation can be involved.

In conclusion, this study maps DNA methylation status of the HLA-DQA1 promoter region and evaluates HLA-DQA1 expression differences between patients with T1D and healthy controls. The study contributes to the epigenetic research, analyses in detail DQA1 promoter methylation, and extends the knowledge about epigenetic modification in T1D susceptible gene. Although we have not proved significant methylation differences between the two groups, detailed DNA methylation status and its correlation with expression of each HLA-DQA1 allele in patients with T1D have been described for the first time.

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Disclosure

The authors declare that they have no financial or commercial conflict of interests.

Author contributions

Marie Cerna is author of the research idea, designed the experiments and performed the publication revision. Elena Silhova participated in organization of the collection of the clinical database. Anna Kotrbova-Kozak designed experiments and participated in publication revision and data analysis. Marta Zajacova participated in laboratory work, data analysis and publication revision. Pavel Cepek participated in laboratory work, data analysis and prepared the publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Relative expression of HLA DQA1*02:01/03 alleles in heterozygotes.

Figure S2. Methylation profile of whole blood leucocytes and monocytes of patients with type 1 diabetes mellitus.

Table S1. DRB1–QAP–DQA1–DQB1 haplotypes in patients with type 1 diabetes mellitus and healthy controls.

Table S2. Expression ratios of two alleles in DQA1 heterozygotes and their comparison between patients with type 1 diabetes mellitus and healthy controls.