Appendix 1

Calculations of qPCR Assays Efficiencies

Calculations of qPCR Assay Efficiencies

Design of qPCR assays requires finding the optimal properties of primers and probes within the constrains determined by actual properties of the DNA sequence of the target region. The high polymorphism of the class II region and the need to prepare both allele-specific assays as well as 'total' assays amplifying all alleles of the gene posed an additional challenge in our study. As a result, some assays used in the study were suboptimal and we had to correct for their lower amplification efficiencies to prevent false-positive observations of differences in the mRNA level between alleles. Furthermore, while analyzing the qPCR readings for certain assays, we had to set the assay-specific thresholds; and some assays targeted a DNA in addition to RNA. All these features were taken into account during calculations used to analyze the results. The following document presents all the calculations and reasoning behind them.

Contents

Abbreviations

cX cT (threshold cycle) of an assay X
tX threshold set for an assay X
EX efficacy of an assay X
X shortcut of the equation $(1+EX)^{cX}$
nX input amount of the template that is recognized by an assay X (amount of template before amplification),
nXd DNA fraction of nX
nXr cDNA fraction of nX (r in nXr stands for RNA)
nX = nXr + nXd
A allele-specific assay, amplifies only the allele A
T total assay, amplifies all alleles of the gene
I intronic assay, amplifies intronic gene region, nId = nTd
R RNA (cDNA) content of the sample; R=[#RNA molecules/(#RNA molecules + #DNA molecules)]
D DNA content of the sample; D=[#DNA molecules/(#RNA molecules + #DNA molecules)]
R+D = 1 (100 %)

1. Assay Properties

1.1. Assay properties

The table below lists individual qPCR assays properties that are important for subsequent calculations: whether an assay targets RNA or DNA, and the threshold that was set for the assay after a qPCR measurement.

DQA1				DQB1				other			
assay	RNA	DNA	threshold	assay	RNA	DNA	threshold	assay	RNA	DNA	threshold
01	+	+	0.35	02	+	-	0.3	DRA	+	-	0.2
02	+	+	0.2	03:01	+	-	0.3	PPIA	+	-[1]	0.35
03	+	+	0.35	03:02,03:03,04	+	-	0.3	DQA2	+	-	0.35
04	+	+	0.35	05	+	+	0.3				
05	+	+	0.35	06	+	+	0.3				
total	+	+	0.35	total	+	-	0.3				
intron	-	+	0.35	intron	-	+	0.3				

[1] The PPIA assay was later on (after we finished all measurements) proclaimed by the manufacturer to possibly off-target DNA. This fact was not considered in our calculations.

1.2. Off-targets

1.2.1. DQA2 gene

A DQA1 total assay amplifies the DQA2 gene as well. The problem was revealed in 01/2015 with a new genome assembly release.
As a solution, we designed DQA2 assay to assess the DQA2 mRNA content in ourn samples. Expression of DQA2 gene was found to be less than 0.01% of the DQA1 gene expression (i.e., at least 14 cycles difference) in all samples and was not included into further calculations.

1.2.2. DQB2 gene

A DQB1 intron assay amplifies the DQB2 gene intron as well. The problem was revealed in 01/2015 with a new genome assembly release.
The primers amplify the DQB2 intron, but there are 2 mismsnatches between the DQB2 gene and the probe (including the one in the central part of the probe), which should prevent the off-target detection by this assay, but this was not experimentally verified.

2. Assumptions

In the calculations, we assume the validity of the following assumptions:

(1) The fluorescence signal (f) during amplification follows the equation fX = nX*[(1+EX)^cX]*kX, where kX is an assay-specific constant describing a correlation between the number of target copies and the fluorescence. We assume that this constant is equal for all assays. If 2 assays (A, B) have the same threshold values, then nA*A = nB*B.

(2) In homozygotes, each nucleic acid copy of an allele is amplified by both, an allele-specific assay and by a total assay, thus nA = nT. More specifically:

- for assays amplifying only RNA, nA = nT = nAr = nTr
- for assays amplifying both RNA and DNA, nA = nT = nAr + nAd = nTr + nTd

(3) Intronic assays measure the amount of genomic DNA, which is the same for each gene, thus nTd = nId and nIDQA1 = nIDQB1.

(4) In heterozygotes, both alleles are represented equally in the genomic DNA. Thus if both allele-specific assays (A, B) amplify DNA, then nAd = nBd = ½*nI.

3. Calculations of Assay Efficiencies

In our setup, two approaches can be used to calculate the assay efficiencies:

- comparing signals from multiple assays targeting the same region
- dilution curve

3.1. Comparing signals from multiple assays targeting the same region (method 1)

This method is based on the assumption (2) that in homozygotes, the number of molecules entering the allele-specific assay and the total assay is the same. Any observed difference in the strength of fluorescent signal must be therefore caused by the differences in the amplification efficiencies between the assays.
Similarly, in heterozygotes, the sum of RNA amplified by allele-specific assays equals to the amount of RNA amplified by the total assay.
This method was used to calculate the efficiencies of:

- intronic assays
- allele-specific assays:
  - for DQA1 gene, if there were at least 3 homozygotes for a particular allele in our study group, we calculated the assay efficiency from the homozygous samples (DQA1*01–*03, *05, *06), otherwise we calculated the efficiency from the heterozygous samples (DQA1*04)
  - for DQB1 gene, we calculated the assay efficiency (all DQB1 alleles) as a mean of efficiency determined from the homozygous samples (if there were at least 3 homozygotes for the allele in our study group) and efficiency determined from the heterozygous samples

3.1.1. Efficiency calculations: intronic assays (EIA, EIB)

Calculation input: known EIA (determined by the); measured cIA, cIB
Calculation output: EIB
Calculation:
$nIA*(1+EIA)^{cIA} = 0{,}35$; $nIB*(1+EIB)^{cIB} = 0.3$
$nIB*(1+EIB)^{cIB} = 6/7*nIA*(1+EIA)^{cIA}$ / nIA = nIB
$(1+EIB)^{cIB} = 6/7*(1+EIA)^{cIA}$ / $\sqrt[cIB]{}$
$1+EIB = (6/7)^{1/cIB}*(1+EIA)^{cIA/cIB}$
$EIB = (6/7)^{1/cIB}*(1+EIA)^{cIA/cIB} - 1$

3.1.2. Efficiency calculations: allele-specific assays

3.1.2.1. Homozygous samples (DQA1, DQB1)

Calculation input: known ET; measured cA, cT
Calculation output: EA
Calculation:
$nA*(1 + EA)^{cA} = tA$, $nT*(1 + ET)^{cT} = tT$
$nA*(1 + EA)^{cA} = (tA/tT)* nT*(1 + ET)^{cT}$
$(1 + EA)^{cA} = (tA/tT)*(nT/nA)*(1 + ET)^{cTn}$
$EA = (nT/nA)^{1/cA}*(tA/tT)^{1/cA}*(1 + ET)^{cT/cA} - 1$
Then:

- for assays, where nT = nA (DQA1*01–*05; DQB1*05,*06):
  $nT/nA = 1$
  $EA = (tA/tT)*(1 + ET)^{cT/cA} - 1$

- for assays, where nT = nA–nI (DQB1*02–*04):
  nT/nA = (nA–nI)/nA = 1 + nI/nA /nI*I = nT*T => nI/nT = T/I
  = 1 + T/I
  = (T+I)/I
  EA = (T+I)/I*(tA/tT)*(1 + ET) $^{cT/cA}$ – 1

3.1.2.2. Heterozygous samples (DQA1)

Calculation input: known EA, ET, I; measured cA, cB, cT, cI
Calculation output: EB
Assumption used in the calculations: for alleles that amplify DNA, nAd = ½*nI (equal representation of both alleles in DNA) and nAr = nA – 1/2*nI
Calculation:

Assay combination	A: DQA1*01–*03, *05, *06 B: DQA1*04
Assay properties	all assays (A, B, T) amplify DNA (tA/tT) is (4/7) for assay A = DQA1*02 is 1 for all other DQA1 assays
EB calculation	nT = nB + nA 1 = nB/nT + nA/nT 1 = T/B + (tA/tT)*T/A 1/T = 1/B + (tA/tT)*1/A 1/B = 1/T – (tA/tT)*1/A = [A – (tA/tT)*T]/(A*T) B = (A*T)/[A – (tA/tT)*T] EB = {(A*T)/[A – (tA/tT)*T]} $^{1/cB}$ – 1

3.1.2.3. Heterozygous samples (DQB1)

Calculation input: known EA, ET, I; measured cA, cB, cT, cI
Calculation output: EB
Assumption used in the calculations: for alleles that amplify DNA, nAd = ½*nI (equal representation of both alleles in DNA) and nAr = nA – 1/2*nI
Calculations:
The calculations depend on the fact whether the allele-specific (A) assays amplify DNA or not:

Assay type	A and B do not amplify DNA	A amplifies DNA, B does not and vice-versa	A and B amplify DNA
Assay combination	A, B: DQB1*02–*04	A: DQB1*05, *06 B: DQB1*02–*04	A, B: DQB1*05, *06
Assay properties	nT = nTr nA = nAr nB = nBr	nT = nTr nA = nAr + nAd, nB = nBr or nA = nAr, nB = nBr + nBd	nT = nTr nA = nAr + nAd nB = nBr + nBd
EB calculation	nT = nB + nA 1 = nB/nT + nA/nT 1 = T/B + T/A 1/T = 1/B + 1/A 1/B = 1/T – 1/A = (A – T)/(A*T)	nT = nAr + nB or nT = nA + nBr nT = nA + nB – 1/2*nI /nT 1 = nA/nT – 1/2*nI/nT + nB/nT 1 = T/A – 1/2*T/I + T/B 1 + 1/2*T/I – T/A = T/B /T 1/T + ½/I– 1/A = 1/B	nT = nAr + nBr = nA + nB – (nAd + nBd) nT = nA + nB – nI /nT 1 = nA/nT + nB/nT – nI/nT 1 = T/A + T/B – T/I /T 1/T = 1/A + 1/B – 1/I

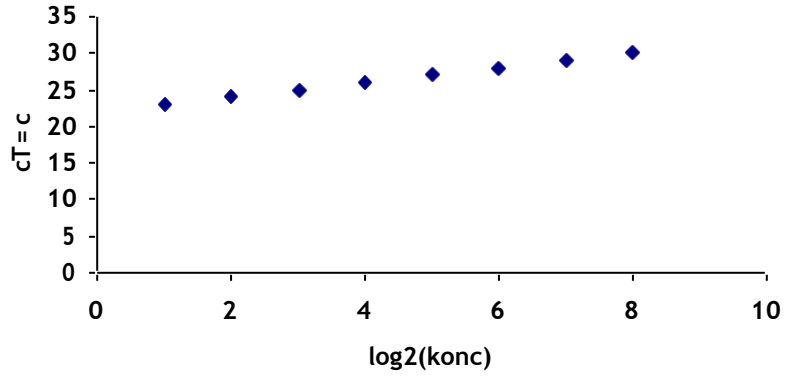
	$B = (A*T)/(A - T)$ $EB = [(A*T)/(A-T)]^{1/cB} - 1$	$B = 1/[1/T + ½/I - 1/A]$ / $^{cB}\sqrt{}$ $EB = 1/[1/T + ½/I - 1/A]^{1/cB} - 1$ $= [(T*A*I)/(A*I - T*I + ½*T*A)]^{1/cB} - 1$	$1/B = 1/T - 1/A + 1/I = (A*I - T*I + T*A)/(T*A*I)$ $B = (T*A*I)/(A*I - T*I + T*A)$ $EB = [(T*A*I)/(A*I - T*I + T*A)]^{1/cB} - 1$ / $^{cB}\sqrt{}$ $EB = 1/(1/T - 1/A + 1/I)^{1/cB} - 1$

3.2. Dilution curve (method 2)

To calculate assay efficiency using the dilution curve, we prepared 2^0 to 2^{-7} dilution series of the 'calibrator' (known efficiency) and the 'test' assays. The Ct values were plotted to the graph against the template concentrations, and the 'tested' assay efficiency was calculated from the slopes of the curves overlying the points for the tested and the calibrator assay.
As the calibrator we chose the DQA1 assay, to which we arbitrarily assigned the efficacy 1.

3.2.1. Advantages of the '2x' dilution series compared to '10x' series

- The dilution mistake is minimized, as we can use the same-size pipette for measuring the cDNA and water solutions
- Compared to the '10x' dilution series, more template concentrations lie within the qPCR range that gives high-precision measurements.



3.2.2. Efficiency calculation from the dilution curve

3.2.2.1. 'Absolute' efficiency

Calculation input: x – template concentration, y – cT at the concentration x
Calculation output: E – 'absolute' assay efficiency
Calculation:

1. Based on the slope (k) definition:
   $k = (y2 - y1)/(x2 - x1)$ (i.e., the slope definition) $/ x2 - x1 = 1$
   $k = c2 - c1$
2. All reactions we used for calculations have the same threshold, thus:
   $n1*(1 + E1)^{c1} = n2*(1 + E2)^{c2}$
3. Assuming that the assay has the same efficacy at all relevant template concentrations:
   $E1 = E2$
   $n1/n2 = (1 + E)^{c2 - c1}$
   $2 = (1 + E)^{c2 - c1} = (1 + E)^{k} \quad / \log_2$
   $\log_2 2 = \log_2 (1 + E)^{k}$
   $1/k = \log_2 (1 + E)$
   $1 + E = 2^{1/k}$
   $E = 2^{1/k} - 1$

3.2.2.2. 'Relative' efficiency

It proved rather difficult to precisely assess an assay efficacy using only the dilution curve. Thus, we aimed to assess relative efficacy of the assays by comparing the slopes of their dilution curves, using the logic below. This method was used to calculate the efficiencies of the intronic assays EIA and EIB.

Calculation input: EA1
Calculation output: EA2
Calculation:

$E1 = 2^{1/k1} - 1, E2 = 2^{1/k2} - 1$
$E2 - E1 = 2^{1/k2} - 1 - 2^{1/k1} + 1$
$E2 - E1 = 2^{1/k2} - 2^{1/k1}$
$E2 = E1 + 2^{1/k2} - 2^{1/k1}$

4. Calculations of Genomic DNA Content in a Sample

We calculated the genomic DNA content of our samples using the formula below.

Calculation input: measured cT, cI

nT = nTr + nTd = nRNA + nDNA; nI = nDNA

Calculation output: DNA content in the input sample

Calculation:

(1)

nT*T = nI*I

(nTr + nTd)*T = nI*I / nI = nTd (both assays target both alleles of the same gene)

(nTr + nTd)*T = nTd*I

(nTr + nTd)/nTd = I/T

nTr/nTd = I/T – 1

(2)

R := nTr/nT, R is RNA (cDNA) content of the sample

D := nTd/nT, D is DNA content of the sample

R+D = 1 (100 %)

nTr/nTd = (nTr/nT)*(nT/nTd) = R/D

According to (1) + (2):

R/D = I/T – 1 / R = 1 – D

(1 – D)/D = I/T – 1

1/D – 1 = I/T – 1

1/D = I/T

D = T/I

R = 1 – D = 1 – T/I

5. Calculations of Allele mRNA Amount

5.1. Allele/Total mRNA ratio

In an ideal situation, in a heterozygote, a sum of mRNA levels measured by allele-specific assays equals to the mRNA level of the 'total' assay. The (nAr + nBr)/nTr ratio was thus used to check whether the calcuations and the actual qPCR reactions were performed correctly.

Calculation input: known EA, ET, EI; measured cA, cT and cI (where applicable)
Calculation output: nAr/nTr (we are interested in allele/total ratio in the RNA fraction of the sample only)
Assumption: tIA = tTA, tIB = tTB
Calculations:
The calculations depend on the fact whether the allele-specific (A) and total (T) assays amplify DNA or not:

assay type	A and T do not amplify DNA	A amplifies DNA, T does not	A and T amplify DNA
assay	DQB1*02–*04	DQB1*05,*06	DQA1*01–*05
nAr/nTr=	= nA/nT	= (nA – nAd)/nT	= (nA–nAd)/(nT–nTd)[1]
	= (tA/tT)*(T/A)	= nA/nT – nAd/nT	= (nA–nAd)/[nT*I/(I-T)] /nAd = nI
		In homozygotes:	*In homozygotes:*
		= (tA/tT)*(T/A) – nI/nT	= (nA/nT–nI/nT)*I/(I–T)
		= (tA/tT)*(T/A) – T/I	= [(tA/tT)*(T/A) – T/I]*I/(I – T)
		= (tA/tT)*(T/A) – D	= [(tA/tT)*(T/A) – D]*(1/R)
		In heterozygotes	*In heterozygotes:*
		= (tA/tT)*(T/A) – ½*nI/nT	= (nA/nT – ½*nI/nT)*I/(I–T)
		= (tA/tT)*(T/A) – ½*T/I	= [(tA/tT)*(T/A) – ½*T/I]*I/(I–T)
		= (tA/tT)*(T/A) – ½*D	= [(tA/tT)*(T/A) – ½*D]*(1/R)

[1](nT–nTd) = nT–nI = nT–nT*(T/I) = nT*(1–T/I) = nT*(I–T)/I ; 1/(nT–nTd) = 1/nT*I/(I-T)

5.2. Allele/DRA mRNA ratio

Before statistical analysis, each measurement was normalized against the endogenous control, the DRA gene.
Calculation input: known EA, EDRA, EI; measured cA, cDRA and cI (where applicable)
Calculation output: nAr/nDRA (we are interested in allele/DRA ratio in mRNA)
Assumption: nDRA = nDRAr (DRA does not amplify DNA)
Calculations:
The calculations depend on the fact whether the allele-specific (A) assays amplify DNA or not:

assay type	A does not amplify DNA	A amplifies DNA
assay	DQB1*02–*04	DQA1*01–*05; DQB1*05,*06
nAr/nDRA=	= nA/nDRA	= (nA – nAd)/nDRA
	= (tA/tDRA)*(DRA/A)	= nA/nDRA – nAd/nDRA
		In homozygotes:
		= (tA/tDRA)*(DRA/A) – nI/nDRA
		= (tA/tDRA)*(DRA/A) –(tI/tDRA)*DRA/I
		In heterozygotes:
		= (tA/tDRA)*(DRA/A) – ½*nI/nDRA
		= (tA/tDRA)*(DRA/A) – ½*(tI/tDRA)*DRA/I

5.3. Allele/PPIA mRNA ratio

We also included a PPIA gene as a classical endogenous control. Before statistical analysis, each measurement was normalized against the PPIA gene.

Calculation input: known EA, EPPIA, EI; measured cA, cPPIA and cI (where applicable)

Calculation output: nAr/nPPIA (we are interested in allele/PPIA ratio in mRNA)

Assumption: nPPIA = nPPIAr (PPIA does not amplify DNA[1])

Calculations:

assay type	A does not amplify DNA	A amplifies DNA
assay	DQB1*02–*04	DQA1*01–*05; DQB1*05,*06
nAr/nPPIA	= nA/nPPIA = (tA/tPPIA)*(PPIA/A)	= (nA – nAd)/nPPIA = nA/nPPIA – nAd/nPPIA *In homozygotes:* = (tA/tPPIA)*(PPIA/A) – nI/nPPIA = (tA/tPPIA)*(PPIA/A) –(tI/tPPIA)*PPIA/I *In heterozygotes:* = (tA/tPPIA)*(PPIA/A) – ½*nI/nPPIA = (tA/tPPIA)*(PPIA/A) – ½*(tI/tPPIA)*PPIA/I

[1]After the measurements were finished, manufacturer disclaimed that an assay may off-target DNA as well. We did not take this into account in our calculations

5.4. DRA/PPIA mRNA ratio

Calculation input: measured cDRA, cPPIA

Calculation output: nDRA/nPPIA

Assumption: nPPIA = nPPIAr, nDRAr = nDRA (PPIA does not amplify DNA)

Calculation: nDRA/nPPIA = (tDRA/tPPIA)*(PPIA/DRA) = 4/7*(PPIA/DRA)

6. Corrections in Repeated Measurements

For each individual in study, we have evaluated mRNA level of multiple (8 to 10) target genes. Sometimes, a need to repeat measurement for a single target gene arose, e.g., due to a mistake in the reaction preparation and subsequent missing signal or high SD. In this case, we did not repeat the qPCR measurement for all genes, instead we repeated just the particular assay together with a 'marker' assay. This enabled us to preserve reproducibility when analyzing the data measured on two different occasions.
Assume we measure a level of genes A, B and C using the respective assays. In case of a problem with a reaction A, we just repeat measurements for the assays A and B. Then we use the amount of the A gene in the second reaction and an inter-reaction difference in the B gene levels to calculate the amount of A in the first reaction. This approach was used wherever we needed to repeat a single assay out of the individuals assay set.

Calculation input: measured cA2, cB1, cB2; known EA, EB
cA1, cA2, cB1, cB2 – cT of an assays A and B in the first (cA1, cB1) or repeated qPCR reaction (cA2, cB2)
Calculation output: cA1
Calculation:
In case the reactions have the same thresholds:
$nA1/nB1 = nA2/nB2$ / (Ass.1)
$A1/B1 = A2/B2$
$A1 = A2*B1/B2$
$(1+EA)^{cA1} = (1+EA)^{cA2}*(1+EB)^{cB2-cB1}$ / $\log_{(1+EA)}$
$cA1 = cA2 + (cB2-cB1)*\log_{(1+EA)}(1+EB)$

Annex 1

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

Marta Zajacova, Anna Kotrbova-Kozak, Pavel Cepek, Marie Cerna

Immunology Letters 167 (2015) 147–154

Immunology Letters 167 (2015) 147–154

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

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

Marta Zajacova, Anna Kotrbova-Kozak, Pavel Cepek, Marie Cerna*

Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Ruska 87, 100 00 Prague, Czech Republic

article info

Article history:
Received 19 June 2015
Received in revised form 7 August 2015
Accepted 12 August 2015
Available online 20 August 2015

Keywords:
DNA methylation
mRNA expression
HLA class II
DQA1
Polymorphism

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.

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, a and þ, 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.

* Corresponding author.
E-mail addresses: marta.zajac17@gmail.com (M. Zajacova), anna.kotrbova@lf3.cuni.cz (A. Kotrbova-Kozak), pa.cepek9@gmail.com (P. Cepek), marie.cerna@lf3.cuni.cz (M. Cerna).

http://dx.doi.org/10.1016/j.imlet.2015.08.006
0165-2478/

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/μl. 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 $NH_4(SO_4)_2$, 6 mM/4 mM $MgCl_2$, 0.5 mM dNTP mix, 0.8 μM of each primer, 1 U/25 μl of Taq and 1 μl/1.5 μl 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 $_{c}$ =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 (synthetized 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

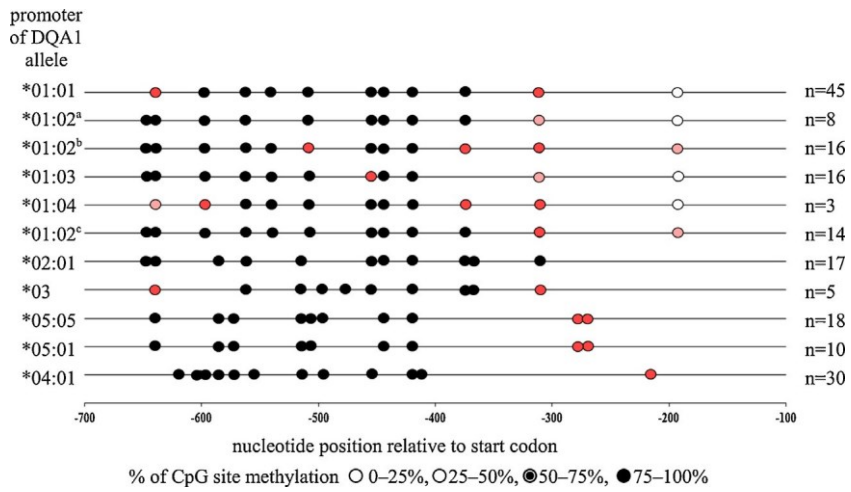


Fig. 1. CpG methylation status of individual CpG sites of DQA1 promoter alleles. According to the polymorphisms present on the sequencing read, sequences were classified into appropriate allelic groups.
The matrix represents an amplicon that contains 9–12 CpG sites (number depends on promoter allele 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.
[a]Linked with DQA1 promoter allele QAP 1.2K.
[b]Linked with DQA1 promoter allele QAP 1.2L.
[c]Linked 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, $p_c = p_{un}*9$).

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±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,

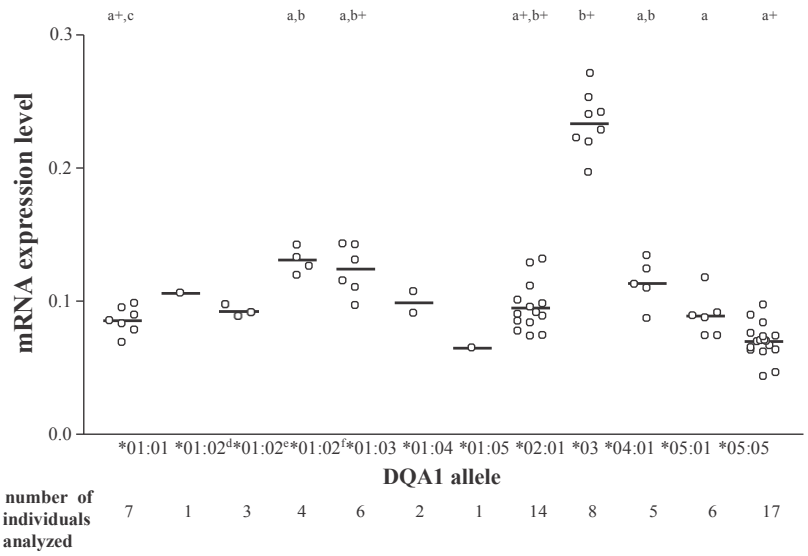


Fig. 2. mRNA expression levels of DQA1 alleles. Expression of DQA1 alleles was determined by qRT-PCR with allele-specific primers and the mean of 3 independent measurements was used for the analysis. Each point in the graph represents normalized value for 1 individual and 1 allele. The horizontal line 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*0401 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 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 a and þ 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

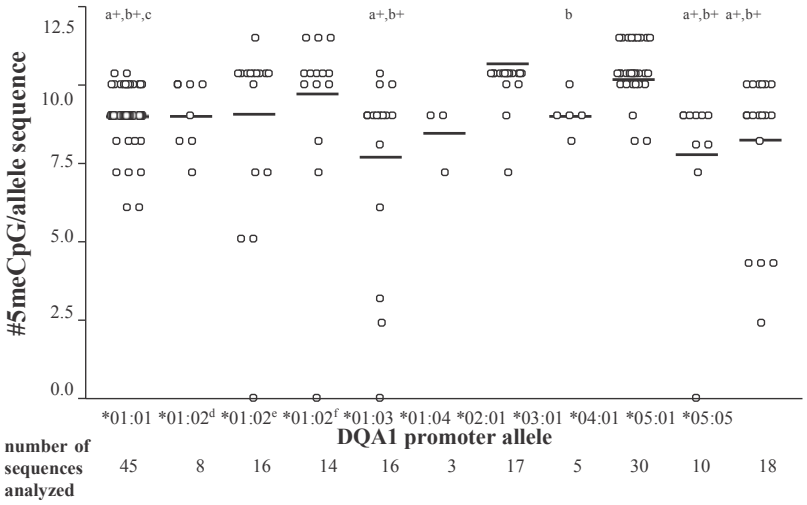


Fig. 3. Overall methylation of DQA1 promoter alleles. According to the polymorphisms present on the sequencing read, sequences were classified into appropriate allelic groups. For each sequence, the total number of methylated CpG sites in the region examined (i.e. overall methylation) was counted. Each 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 +93 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 $(A/T)_{n\geq 4}$ 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.

Acknowledgments

This survey was funded by the Research program of Charles University in Prague: PRVOUK P31 – initial stadia of diabetes, obesity and other metabolic diseases and 260,165/SVV/2015 – diabetes mellitus, a complex metabolic disorder with many aspects. A.K.K. gratefully acknowledges support of the Charles University through University Research Centre program UNCE 204,015. We thank Vera Lamborova for her excellent technical assistance.

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.

References

[1] W. Reith, S. LeibundGut-Landmann, J.-M. Waldburger, Regulation of MHC class II gene expression by the class II transactivator, Nat. Rev. Immunol. 5 (2005) 793–806, http://dx.doi.org/10.1038/nri1708.
[2] M. Cerna, Genetics of autoimmune diabetes mellitus, Wien. Med. Wochenschr. 158 (2008) 2–12.
[3] L.M. Schwiebert, A. Hughes, D.S. Monos, S.J. Ono, HLA-DRA promoter polymorphism and diversity generation within the immune system, Hum. Genet. 99 (1997) 801–805.
[4] C. Perfetto, M. Zacheis, D. McDaid, J.W. Meador, B.D. Schwartz, Polymorphism in the promoter region of HLA-DRB genes, Hum. Immunol. 36 (1993) 27–33.
[5] G. Del Pozzo, C. Perfetto, M.N. Ombra, G.Z. Ding, J. Guardiola, A. Maffei, DNA polymorphisms in the 5′-flanking region of the HLA-DQA1 gene, Immunogenetics 35 (1992) 176–182.
[6] E. Morzycka-Wroblewska, J.I. Harwood, J.R. Smith, M.F. Kagnoff, Structure and evolution of the promoter regions of the DQA genes, Immunogenetics 37 (1993) 364–372.
[7] L.C. Andersen, J.S. Beaty, J.W. Nettles, C.E. Seyfried, G.T. Nepom, B.S. Nepoom, Allelic polymorphism in transcriptional regulatory regions of HLA-DQB genes, J. Exp. Med. 173 (1991) 181–192.
[8] G. Brünnler, J. Haas, L. Fan, M. Petzl-Erler, A. Volgger, Z. Yao, et al., DQA1 promoter polymorphism: 12th international histocompatibility workshop study, Proc. Twelfth Int. Histocompat. Work. Conf. Vol I Work (1997) 171–175.
[9] N.A. Hosken, K. Shibuya, A.W. Heath, K.M. Murphy, A.O. Garra, The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-αβ-transgenic model, J. Exp. Med. 182 (1995) 20–22.
[10] L. DiMolfetto, H.A. Neal, A. Wu, C. Reilly, D. Lo, The density of the class II MHC T cell receptor ligand influences IFN-gamma/IL-4 ratios in immune responses in vivo, Cell Immunol. 183 (1998) 70–79, http://dx.doi.org/10.1006/cimm.1997.1231.
[11] I.G. Ovsyannikova, J.E. Ryan, R.M. Jacobson, R.A. Vierkant, S. Pankratz, G.A. Poland, Human leukocyte antigen and interleukin 2, 10 and 12p40 cytokine responses to measles: is there evidence of the hla effect? Cytokine 36 (2007) 173–179.
[12] A.C. Britten, C.H. Mijovic, A.H. Barnett, M.A. Kelly, Differential expression of HLA-DQ alleles in peripheral blood mononuclear cells: alleles associated with susceptibility to and protection from autoimmune type 1 diabetes, Int. J. Immunogenet. 36 (2009) 47–57, http://dx.doi.org/10.1111/j.1744-313X2008.00823.x.
[13] H. Donner, C. Seidl, H. Rau, J. Herwig, E. Seifried, K.H. Usadel, et al., Unbalanced amounts of HLA-DQA1 allele mRNA: DQA1*03 shows high and DQA1*0501 low amounts of mRNA in heterozygous individuals, Eur. J. Immunogenet. 29 (2002) 321–330.
[14] S. Fernandez, R. Wassmuth, I. Knerr, C. Frank, J.P. Haas, Relative quantification of HLA-DRA1 and -DQA1 expression by real-time reverse transcriptase–polymerase chain reaction (RT-PCR), Eur. J. Immunogenet. 30 (2003) 141–148.
[15] E. Morzycka-Wroblewska, A. Munshi, M. Ostermayer, J.I. Harwood, M.F. Kagnoff, Differential expression of HLA-DQA1 alleles associated with promoter polymorphism, Immunogenetics 45 (1997) 163–170.
[16] R. Vincent, P. Louis, C. Gongora, I. Papa, J. Clot, J.F. Eliaou, Quantitative analysis of the expression of the HLA-DRB genes at the transcriptional level by competitive polymerase chain reaction, J. Immunol. 156 (1996) 603–610.
[17] Y. Zhang, C. Rohde, S. Tierling, T.P. Jurkowski, C. Bock, D. Santacruz, et al., DNA methylation analysis of chromosome 21 gene promoters at single base pair and single allele resolution, PLoS Genet. 5 (2009) e1000438, http://dx.doi.org/10.1371/journal.pgen.1000438.
[18] K. Kerkel, A. Spadola, E. Yuan, J. Kosek, L. Jiang, E. Hod, et al., Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation, Nat. Genet. 40 (2008) 904–908, http://dx.doi.org/10.1038/ng.174.
[19] C.-K. Lee, Y. Shibata, B. Rao, B.D. Strahl, J.D. Lieb, Evidence for nucleosome depletion at active regulatory regions genome-wide, Nat. Genet. 36 (2004) 900–905, http://dx.doi.org/10.1038/ng1400.
[20] B.D. Strahl, C.D. Allis, The language of covalent histone modifications, Nature 403 (2000) 41–45.
[21] K. Raghavan, H.J. Ruskin, D. Perrin, F. Goasmat, J. Burns, Computational micromodel for epigenetic mechanisms, PLoS One 5 (2010) e14031, http://dx.doi.org/10.1371/journal.pone.0014031.
[22] R. Jaenisch, A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals, Nat. Genet. 33 (2003) 245–254, http://dx.doi.org/10.1038/ng1089.
[23] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.
[24] A. Maffei, G.D. Pozzo, A. Prisco, M. Ciullo, P.E. Harris, E.F. Reed, et al., Polymorphism in the 5′ terminal region of the mRNA of HLA-DQA1 gene: identification of four groups of transcripts and their association with polymorphism in the alpha 1 domain, Hum. Immunol. 53 (1997) 167–173.
[25] B.S. Constant, C. Pfeiffer, A. Woodard, T. Pasqualini, K. Bottomly, Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells, J. Exp. Med. 182 (1995) 5.
[26] P.R. Rogers, M. Croft, Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance, J. Immunol. 163 (2014) 1205–1213.
[27] E.K. Shiner, B.C. Holbrook, M.A. Alexander-Miller, CD4+ T cell subset differentiation and avidity setpoint are dictated by the interplay of cytokine and antigen mediated signals, PLoS One 9 (2014) e100175, http://dx.doi.org/10.1371/journal.pone.0100175.
[28] R.A. Gottschalk, E. Corse, J.P. Allison, TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo, J. Exp. Med. 207 (2010) 1701–1711, http://dx.doi.org/10.1084/jem.20091999.
[29] G. Iezzi, I. Sonderegger, F. Ampenberger, N. Schmitz, B.J. Marsland, M. Kopf, CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4 ‰ T cells, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1–6.
[30] J.X. She, Susceptibility to type I diabetes: HLA-DQ and DR revisited, Immunol. Today 17 (1996) 323–329.
[31] J.J. Yunis, N. Mobini, E.J. Yunis, C.A. Alper, R. Deulofeut, A. Rodriguez, et al., Common major histocompatibility complex class II markers in clinical variants of cicatricial pemphigoid, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 7747–7751.

[32] A.S. Louka, L.M. Sollid, HLA in coeliac disease: unravelling the complex genetics of a complex disorder, Tissue Antigens 61 (2003) 105–117.
[33] L.A. Otten, V. Steimle, Quantitative control of MHC class II expression by the transactivator CIITA, Eur. J. Immunol. 28 (1998) 473–478.
[34] J.P. Ting, J. Trowsdale, C. Hill, N. Carolina, F. Allcock, Genetic control of MHC class II expression, Cell 109 (2002) S21–S33.
[35] R.J. Klose, A.P. Bird, Genomic DNA methylation: the mark and its mediators, Trends Biochem. Sci. 31 (2006) 89–97, http://dx.doi.org/10.1016/j.tibs.2005.12.008.
[36] M. Weber, I. Hellmann, M.B. Stadler, L. Ramos, S. Pääbo, M. Rebhan, et al., Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome, Nat. Genet. 39 (2007) 457–466, http://dx.doi.org/10.1038/ng1990.
[37] R.J. Klose, S.A. Sarraf, L. Schmiedeberg, S.M. McDermott, I. Stancheva, A.P. Bird, DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG, Mol. Cell 19 (2005) 667–678, http://dx.doi.org/10.1016/j.molcel.2005.07.021.
[38] T. Clouaire, J.I. de Las Heras, C. Merusi, I. Stancheva, Recruitment of MBD1 to target genes requires sequence-specific interaction of the MBD domain with methylated DNA, Nucleic Acids Res. 38 (2010) 4620–4634, http://dx.doi.org/10.1093/nar/gkq228.
[39] A. Prokhortchouk, B. Hendrich, H. Jørgensen, A. Ruzov, M. Wilm, G. Georgiev, et al., The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor, Genes Dev. 15 (2001) 1613–1618, http://dx.doi.org/10.1101/gad.198501.
[40] P. Majumder, J.M. Boss, DNA methylation dysregulates and silences the HLA-DQ locus by altering chromatin architecture, Genes Immunol. 12 (2011) 291–299, http://dx.doi.org/10.1038/gene.2010.77.
[41] P. Majumder, J.A. Gomez, B.P. Chadwick, J.M. Boss, The insulator factor CTCF controls MHC class II gene expression and is required for the formation of long-distance chromatin interactions, J. Exp. Med. 205 (2008) 785–798, http://dx.doi.org/10.1084/jem.20071843.

Marta Zajacova was graduated from Biology (2008 bachelor's degree) and Immunology (2010 master's) at the Faculty of Science, Charles University in Prague. In her diploma thesis she focused on epigenetic regulation of HLA class II genes. Currently she is a PhD student at the Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, where she continues to explore the topic of HLA epigenetics. She has published one article in impacted journal.

Anna Kotrbova-Kozak is an assistant professor at the Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague. She graduated from Institute of Chemical Technology, Prague, specialization in Biochemistry (1999 MSc, 2007 PhD). In 2001–2002 she worked as researcher at the Research Institute of Innovative Technology for the Earth in Kyoto, Japan. Her research interests include different genotyping techniques, copy number variation analysis, genome-wide association studies and epigenetic influence on metabolic diseases such as diabetes mellitus.

Pavel Cepek completed his bacherol's studies in biochemistry (2010, Charles University in Prague) and gained master's degree in molecular biology and genetics of eukaryotes (2012, Charles University). He started his PhD studies in 2012 at the Third Faculty of Medicine (Charles University in Prague), where he continued in his master's thesis named Epigenetic regulation of HLA class II genes and their role in autoimmune diseases. In 2014 he started to work at the Institute of Rheumatology (Prague), where he concentrates on the expression of HSP 70 genes, miRNA and their relationship to autoimmune diseases. He has published one article.

Marie Cerna Associate Professor of Medical Biology, is a head of the Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague. She graduated from the First Faculty of Medicine, Charles University in Prague (1989 MD, 1994 PhD). She finished her Postdoctoral research training at the University of Texas Southwestern Medical School in Dallas, U.S.A. Dr. Marie Cerna is an expert on immunogenetics. Her research centers on the DNA analyses of human major histocompatibility system (HLA). She possesses expertise on etiopathogenesis of autoimmune diseases such as type 1 diabetes mellitus and rheumatologic diseases.

Appendix A: Supplemental Tables

Supplemental Table A.1. Sequences and properties of bisulfite sequencing and RT-PCR primers and probes. [a] Even though the promoter region amplified by the DQA1*04:01 assay is shorter, the omitted area is free of CpG dinucleotides (see also Fig. 1), therefore it is possible to compare overall methylation of this allele with overall methylation of other promoter alleles.
[b] Primers adopted from the sequences published by Fernandez et al., 2003. The fluorogenic probes are FAM-labelled at the 5' end and double-quenched by Iowa Black at the 3' end and by ZEN internal quencher at 9 nucleotide distance from the dye.
[c] metF_04-DQA was used as a forward primer in both, first and second, PCR reaction to amplify DQA1*04 allele of DQA1 gene

Assay	Primer name	Sequence (5′→3′)	Length	Tm (°C)	Amplicon length (bp)	location
Bisulfite sequencing primers						
DQA1_all	metF1-DQA	GGTTGTAAGTTAGAATATTTTGAAGGATG	29	63	643	-729 – -87
	metR1-DQA	CAAACCAAACCCTACCAAATCA	22	58		
	metF2-DQA	AGGTTGTTTAGAAATGTTTATTTTTGG	27	59	548	-673 – -126
	metR2-DQA	AAAATCCCCTATAATAACATCTCAATTAC	29	62		
DQA1*04	metF_04-DQA[c]	TTATTTATTTACGAGGTTGTTTAGAAATG	29	53	572	-686 – -115
	metR1_04-DQA	AACTAATTAAAAAAACTCCCCTATAATAACAC	31	53		
	metR2_04-DQA	TTTAACAAAAAAATCCCCTAATTATAAC	28	52	501	-686– -186[a]
Real-time PCR primers and probes						
DQA1*01	DQA01F	GAAGGAGACTGCCTGGCG	18	53	106	exon 2
	DQA01R	CATGATGTTCAAGTTGTGTTTTGC	24	54		
	DQA01FAIB	CCTGCGGGTCAAAACCTCCAAATTTG	26	66		
DQA1*02	DQA02F	TTACGGTCCCTCTTGCCAGTT	21	55	124	exon 2
	DQA02R	TTGCGGGTCAAATCTAAGTCTGT[b]	23	55		
	DQA0203FAIB	CCACATAGAACTCCTCGTCTCCATCAAATTCAT	33	66		
DQA1*03	DQA03F	GGTCCCTCTGGGCAGTACAG	20	53	127	exon 2
	DQA03R	CAAATTGCGGGTCAAATCTTCT[b]	22	55		
	DQA0203FAIB	CCACATAGAACTCCTCGTCTCCATCAAATTCAT	33	66		
DQA1*04	DQA04F	GTACACCCATGAATTTGATGGAGAC	25	55	154	exon 2
	DQA04R	CAGGATGTTCAAGTTGTGTTTTGTC	25	55		
	DQA0405FAIB	ACTGTCTGGTGTTTGCCTGTTCTCAGACAA[b]	30	65		
DQA1*05	DQA05F	GATGAGCAGTTCTACGTGGACCC	23	54	152	exon 2
	DQA05R	GTAGAGTTGGAGCGTTTAATCAGAC	25	53		
	DQA0405FAIB	ACTGTCTGGTGTTTGCCTGTTCTCAGACAA[b]	30	65		
DQA1total	DQAtotalF	TACAGCTCAGAACAGCAACTGC	22	53	126	exon 1
	DQAtotalR	CCCACAATGTCTTCACCTCCA	21	55		
	DQAtotalFAIB	CTTTGTTTAGGATCATCCTCTTCCCAAGGC	30	65		
DQA1intron	DQAintrF	GTTGCCCGTTTCTTTCTCTCA[b]	21	54	80	intron 1
	DQAintrR	TGGACTCCTTTACCCACTCCC[b]	21	55		
	DQAintrFAIB	ACCTGTGCCAGTTCCCATGTGGAAAT	26	64		
DRA	DRAF	GGACAAAGCCAACCTGGAAA[b]	20	54	120	exon 2–3
	DRAR	AGGACGTTGGGCTCTCTCAG[b]	20	53		
	DRA_FAIB	CAACTATACTCCGATCACCAATGTACCTCCAGAG	34	65		

Supplemental Table A.2. Differences in relative expression of DQA1 alleles. All expression data of DQA1 alleles were normalized to the DRA expression level. p-values that remained statistically significant after Bonferroni correction ($p_c=p_{un}*9$) are highlighted in bold.
[a] linked with DQA1 promoter allele QAP 1.2L
[b] linked with DQA1 promoter allele QAP 1.4
[c] p_c=NS by two-tailed Mann-Whitney test with 95% CI and after Bonferroni correction
[d] p_c<0.05 by two-tailed Mann-Whitney test with 95% CI and after Bonferroni correction
[e] p_c<0.005 by two-tailed Mann-Whitney test with 95% CI and after Bonferroni correction

	DQA1 allele								
DQA1 allele 2	*01:01 (n=7)	*01:02[a] (n=3)	*01:02[b] (n=4)	*01:03 (n=6)	*02:01 (n=14)	*03 (n=8)	*04:01 (n=5)	*05:01 (n=6)	*05:05 (n=17)
*01:01	-	NS	0.0061 [c]	0.0023 [d]	NS	0.0003 [e]	0.0177 [c]	NS	0.0133 [c]
*01:02[a]	-	-	NS	0.0476 [c]	NS	0.0121 [c]	NS	NS	0.0199 [c]
*01:02[b]	-	-	-	NS	0.0126 [c]	0.0040 [d]	NS	0.0095 [c]	0.0027 [d]
*01:03	-	-	-	-	0.0119 [c]	0.0007 [d]	NS	0.0152 [c]	0.0005 [e]
*02:01	-	-	-	-	-	0.0002 [e]	NS	NS	0.0001 [e]
*03	-	-	-	-	-	-	0.0016 [d]	0.0007 [d]	<0.0001 [e]
*04:01	-	-	-	-	-	-	-	NS	0.0017 [d]
*05:01	-	-	-	-	-	-	-	-	0.0087 [c]

Supplemental Table A.3. Expression ratio of DQA1 alleles. For each allele, the mean relative expression level was calculated (mean allele expression). To obtain the expression ratio of two alleles, the mean expression of one allele (in this table in the title row) was divided by the mean expression of the second allele (title column). Data for allele pairs whose expression proved to differ significantly (Fig. 2, Supplemental Table A.2) are highlighted in bold.
[a] linked with DQA1 promoter allele QAP 1.2L
[b] linked with DQA1 promoter allele QAP 1.4

	DQA1 allele								
	*01:01	*01:02[a]	*01:02[b]	*01:03	*02:01	*03	*04:01	*05:01	*05:05
mean allele expression	0.081	0.088	0.127	0.120	0.091	0.235	0.109	0.085	0.066
(SEM)	(0.004)	(0.003)	(0.006)	(0.008)	(0.005)	(0.008)	(0.008)	(0.006)	(0.003)
DQA1 allele *01:01	1	1.08	1.57	1.48	1.12	2.88	1.34	1.04	0.81
DQA1 allele *01:02[a]	0.92	1	1.44	1.36	1.03	2.67	1.24	0.97	0.75
DQA1 allele *01:02[b]	0.64	0.69	1	0.94	0.72	1.85	0.86	0.67	0.52
DQA1 allele *01:03	0.68	0.73	1.06	1	0.76	1.96	0.91	0.71	0.55
DQA1 allele *02:01	0.89	0.97	1.40	1.32	1	2.58	1.20	0.93	0.73
DQA1 allele *03	0.34	0.37	0.54	0.51	0.39	1	0.46	0.36	0.28
DQA1 allele *04:01	0.74	0.81	1.17	1.10	0.83	2.16	1	0.78	0.61
DQA1 allele *05:01	0.95	1.04	1.49	1.41	1.07	2.76	1.28	1	0.78
DQA1 allele *05:05	1.23	1.33	1.92	1.82	1.38	3.56	1.66	1.29	1

Supplemental Table A.4. Expression ratio of DQA1 alleles in heterozygotes. For each heterozygous individual in our sample, the expression ratio of the two alleles was calculated by dividing expression of one allele (in this table listed as first) by expression of the second allele (listed as second). When data from multiple individuals with the same allelic combinations were available, the mean value for each combination was calculated, and used.
[a] linked with DQA1 promoter allele QAP 1.2K
[b] linked with DQA1 promoter allele QAP 1.2L
[c] linked with DQA1 promoter allele QAP 1.4

DQA1 allele combination		expression ratio (allele1/allele2)		
(allele1, allele2)	n	mean	range	SD
*02:01, *01:01	3	1.10	1.02–1.23	0.09
*03, *01:03	2	2.33	1.76–2.90	0.57
*04:01,*02:01	2	1.12	1.04–1.19	0.07
*01:04, *05:01	2	1.16	1.12–1.21	0.05
*01:01, *05:05	2	1.32	1.10–1.54	0.22
*01:02[c], *05:05	2	1.82	1.80–1.83	0.02
*01:03, *05:05	2	1.73	1.67–1.80	0.06
*02:01, *05:05	3	1.40	1.23–1.61	0.16
*03, *05:05	2	4.84	4.40–5.28	0.44
*04:01, *05:05	3	1.85	1.73–1.92	0.08
Genotypes found in only one individual				
*03, *01:01		2.44	–	–
*03, *01:02[c]		1.70	–	–
*02:01, *01:02[b]		1.20	–	–
*03, *01:02[b]		2.88	–	–
*01:03, *02:01		1.59	–	–
*03, *02:01		3.01	–	–
*01:02[c], *05:01		1.65	–	–
*01:03, *05:01		1.27	–	–
*02:01, *05:01		1.08	–	–
*01:02[a], *05:05		1.54	–	–
*01:02[b], *05:05		1.34	–	–
*01:05, *05:05		0.92	–	–

Supplemental Table A.5. Total relative DQA1 expression – measured. Expression data of DQA1 alleles were normalized to the DRA expression level. The graph shows the sum of expression levels of both alleles (DQA1 allele 1 and DQA1 allele 2) present in an individual. In heterozygotes whose alleles' expression is indiscernible by our approach (i.e. DQA1*010x/*010y and DQA1*05:01/*05:05 heterozygotes), normalized DQA1 total expression value was used. If data from multiple individuals with given allelic combinations were available, the mean value for each combination is shown. Dash (–) marks allele combinations that are not present in our sample.
[a] linked with DQA1 promoter allele QAP 1.2K
[b] linked with DQA1 promoter allele QAP 1.2L
[c] linked with DQA1 promoter allele QAP 1.4

DQA1 allele 2 \ DQA1 allele 1	*01:01	*01:02[a]	*01:02[b]	*01:02[c]	*01:03	*01:04	*01:05	*02:01	*03	*04:01	*05:01	*05:05
*01:01	0.18	–	–	–	0.28	0.19	–	0.17	0.32	–	–	0.14
*01:02[a]	–	–	–	–	–	–	–	–	–	–	–	0.17
*01:02[b]	–	–	–	–	–	–	–	0.18	0.33	–	–	0.16
*01:02[c]	–	–	–	–	–	–	–	–	0.38	–	0.18	0.19
*01:03	0.28	–	–	–	–	–	–	0.18	0.36	–	0.19	0.22
*01:04	0.19	–	–	–	–	–	–	–	–	–	0.17	–
*01:05	–	–	–	–	–	–	–	–	–	–	–	0.13
*02:01	0.17	–	0.18	–	0.18	–	–	0.16	0.34	0.20	0.18	0.18
*03	0.32	–	0.33	0.38	0.36	–	–	0.34	–	–	–	0.25
*04:01	–	–	–	–	–	–	–	0.20	–	–	–	0.17
*05:01	–	–	–	0.18	0.19	0.17	–	0.18	–	–	0.17	0.14
*05:05	0.14	0.17	0.16	0.19	0.22	–	0.13	0.18	0.25	0.17	0.14	–

Supplemental Table A.6. Total relative DQA1 expression – predicted. Expression data of DQA1 alleles were normalized to the DRA expression level. For each allele, the mean relative expression level was calculated (allele mean). The mean expression levels of two alleles were added up to calculate predicted total DQA1 mRNA level. Data for allelic combinations not present in our study sample are denoted in smaller font.

[a] linked with DQA1 promoter allele QAP 1.2K
[b] linked with DQA1 promoter allele QAP 1.2L
[c] linked with DQA1 promoter allele QAP 1.4

		DQA1 allele 1											
		*01:01	*01:02[a]	*01:02[b]	*01:02[c]	*01:03	*01:04	*01:05	*02:01	*03	*04:01	*05:01	*05:05
	allele mean	0.081	0.102	0.088	0.127	0.120	0.091	0.061	0.095	0.235	0.109	0.085	0.066
*01:01	0.081	0.16	0.18	0.17	0.21	0.20	0.18	0.14	0.17	0.32	0.19	0.17	0.15
*01:02[a]	0.102	0.18	0.20	0.19	0.23	0.22	0.20	0.16	0.19	0.34	0.21	0.19	0.17
*01:02[b][c]	0.088	0.17	0.19	0.18	0.22	0.21	0.18	0.15	0.18	0.32	0.20	0.17	0.15
*01:02	0.127	0.21	0.23	0.22	0.25	0.25	0.22	0.19	0.22	0.36	0.24	0.21	0.19
*01:03	0.120	0.20	0.22	0.21	0.25	0.24	0.22	0.18	0.21	0.36	0.23	0.21	0.19
*01:04	0.095	0.18	0.20	0.18	0.22	0.22	0.19	0.16	0.19	0.33	0.20	0.18	0.16
*01:05	0.061	0.14	0.16	0.15	0.19	0.18	0.16	0.12	0.15	0.30	0.17	0.15	0.13
*02:01	0.091	0.17	0.19	0.18	0.22	0.21	0.19	0.15	0.18	0.33	0.20	0.18	0.16
*03	0.235	0.32	0.34	0.32	0.36	0.36	0.33	0.30	0.33	0.47	0.34	0.32	0.30
*04:01	0.109	0.19	0.21	0.20	0.24	0.23	0.20	0.17	0.20	0.34	0.22	0.19	0.18
*05:01	0.085	0.17	0.19	0.17	0.21	0.21	0.18	0.15	0.18	0.32	0.19	0.17	0.15
*05:05	0.066	0.15	0.17	0.15	0.19	0.19	0.16	0.13	0.16	0.30	0.18	0.15	0.13

DQA1 allele 2

Supplemental Table A.7. Differences in overall methylation of DQA1 promoter alleles. For each sequence, total number of methylated CpG sites in the region examined (i.e. overall methylation) was counted. According to the polymorphisms present on the sequencing read, sequences were classified into appropriate allelic groups and differences in overall methylation between allelic groups were tested. Uncorrected p-values of all comparisons performed are shown. p-values that remained statistically significant after correction are highlighted in bold.

[a] linked with DQA1 promoter allele QAP 1.2K
[b] linked with DQA1 promoter allele QAP 1.2L
[c] linked with DQA1 promoter allele QAP 1.4
[d] p_c= NS by two-tailed Mann-Whitney test with 95% CI and after Bonferroni correction
[e] p_c<0.05 by two-tailed Mann-Whitney test with 95% CIand after Bonferroni correction
[f] p_c<0.005 by two-tailed Mann-Whitney test with 95% CI and after Bonferroni correction

	promoter allele of DQA1									
	*01:01 (n=45)	*01:02[a] (n=8)	*01:02[b] (n=16)	*01:02[c] (n=14)	*01:03 (n=16)	*02:01 (n=17)	*03 (n=5)	*04:01 (n=30)	*05:01 (n=10)	*05:05 (n=18)
*01:01	-	NS	0.0412 [d]	0.0041 [e]	NS	<0.0001 [f]	NS	<0.0001 [f]	NS	NS
*01:02[a]	-	-	NS	NS	NS	0.0027 [d]	NS	0.0011 [d]	NS	NS
*01:02[b]	-	-	-	NS	0.0364 [d]	NS	NS	NS	NS	0.0217 [d]
*01:02[c]	-	-	-	-	0.0077 [d]	NS	NS	NS	0.0092 [d]	0.0069 [d]
*01:03	-	-	-	-	-	0.0001 [f]	NS	<0.0001 [f]	NS	NS
*02:01	-	-	-	-	-	-	0.0086 [d]	NS	0.0002 [f]	<0.0001 [f]
*03	-	-	-	-	-	-	-	0.0047 [e]	NS	NS
*04:01	-	-	-	-	-	-	-	-	<0.0001 [f]	<0.0001 [f]
*05:01	-	-	-	-	-	-	-	-	-	NS

promoter allele of DQA1

Annex 2

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

Pavel Cepek, Marta Zajacova, Anna Kotrbova-Kozak, Elena Silhova and Marie Cerna

Immunology 148 (2016) 150–159

Immunology
The Journal of cells, molecules, systems and technologies

IMMUNOLO GY ORIGINAL ARTICLE

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

Pavel Cepek,[1] Marta Zajacova,[1] Anna Kotrbova-Kozak,[1] Elena Silhova[2] and Marie Cerna[1]

[1]*Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Prague, and* [2]*University Hospital Kralovske Vinohrady, Prague, Czech Republic*

doi:10.1111/imm.12593
Received 27 November 2015; revised 29 January 2016; accepted 4 February 2016.
Correspondence: M. Cerna, Department of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Ruska 87, 100 00 Prague, Czech Republic.
Email: marie.cerna@lf3.cuni.cz
Senior author: Marie Cerna

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_{corr} = 0·041; DRA normalization, P_{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 *b*-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 protein 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-*b*-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-*b*-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 ($\alpha = 0{\cdot}05$).

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 5^0-GGT TGT AAG TTA GAA TAT TTT GAA GGA TG-3^0 and R1 5^0-CAA ACC AAA CCC TAC CAA ATC A-3^0 for the first PCR; and F2 5^0-AGG TTG TTT AGA AAT GTT TAT TTT TGG-3^0 and R2 5^0-AAA ATC CCC TAT AAT AAC ATC TCA ATT AC-3^0 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* DH5α 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 m$_M$) and confirmed by colony PCR

Table 1. Characteristics of study subjects

Group	Patients with type 1 diabetes mellitus[1] (n = 61)	Healthy controls[2] (n = 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)	–

[1]Individuals with overt hyperglycaemia and at least one of specific autoantibodies.
[2]Individuals 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 B_{IOEDIT} 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_{TM} 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_{TM} 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 $Elute_{TM}$ 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 $Taqman_{TM}$ probes and primers. All samples were measured in triplicate and contained 200 nM of probe, 300 nM of each primer and 1**9** 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

$G_{RAPHPAD\ PRISM}$ 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 α = 0·05. Statistically significant results were corrected using Bonferroni correction (P multiplied by number of promoter alleles tested, $P_{corr} = P_{uncorr}$ *10). Significant differences between allele frequencies of patients with T1D and healthy controls were determined using chi-square test (α = 0 05) with Bonferroni correction ($P_{corr} = P_{uncorr}$ *10). Differences between individual methylation positions were calculated using Fisher's exact test with α = 0·05 with Bonferroni correction ($P_{corr} = P_{uncorr}$ *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 α = 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 ($P_{corr} = P_{uncorr}$ *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_{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_{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

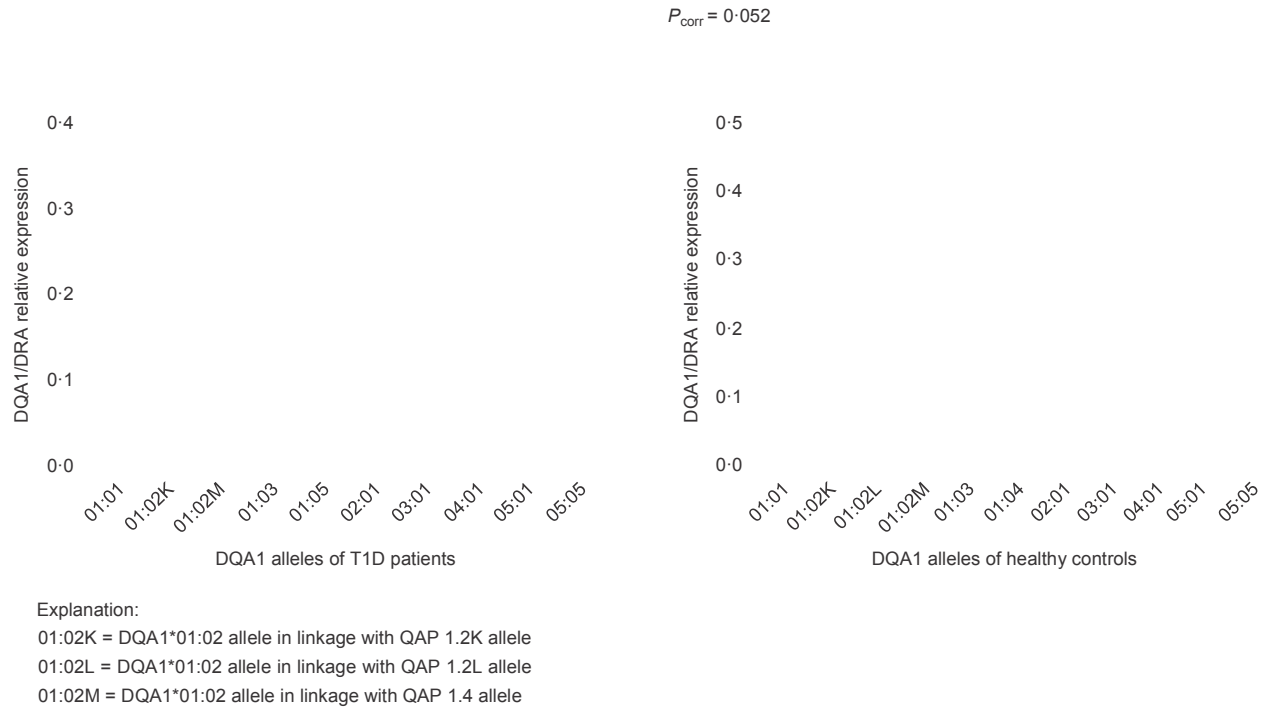


Figure 1. Relative expression of individual DQA1 alleles normalized against HLA-DRA. Each point in the graph represents normalized value for one individual and one allele. The horizontal line indicates mean relative expression of the allele. Mean values with standard error of the mean are indicated. 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_{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 (α = 0·05) followed by Bonferroni correction ($P_{corr} = P_{uncorr}$ *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 α = 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_{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

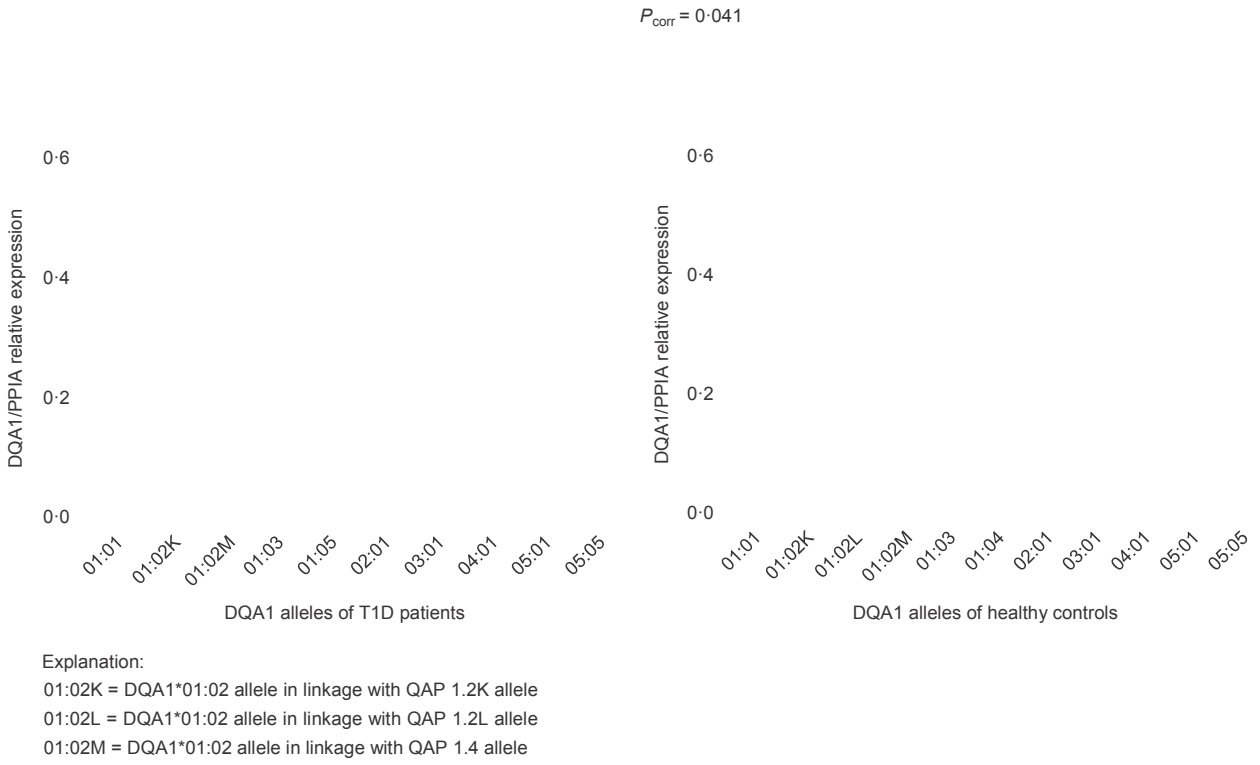


Figure 2. Relative expression of individual DQA1 alleles normalized against peptidylprolyl isomerase A (PPIA). Each point in the graph represents normalized value for one individual and one allele. The horizontal line indicates mean relative expression of the allele. Mean values with standard error of the mean are indicated. DQA1*02:01 was significantly more expressed in healthy controls than in patients with type 1 diabetes mellitus (T1D) (P_{corr} = 0·041). HLA-DQA1*0501 expression was elevated in patients with T1D compared with healthy controls, but significance was lost after correction (P_{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*0603 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.

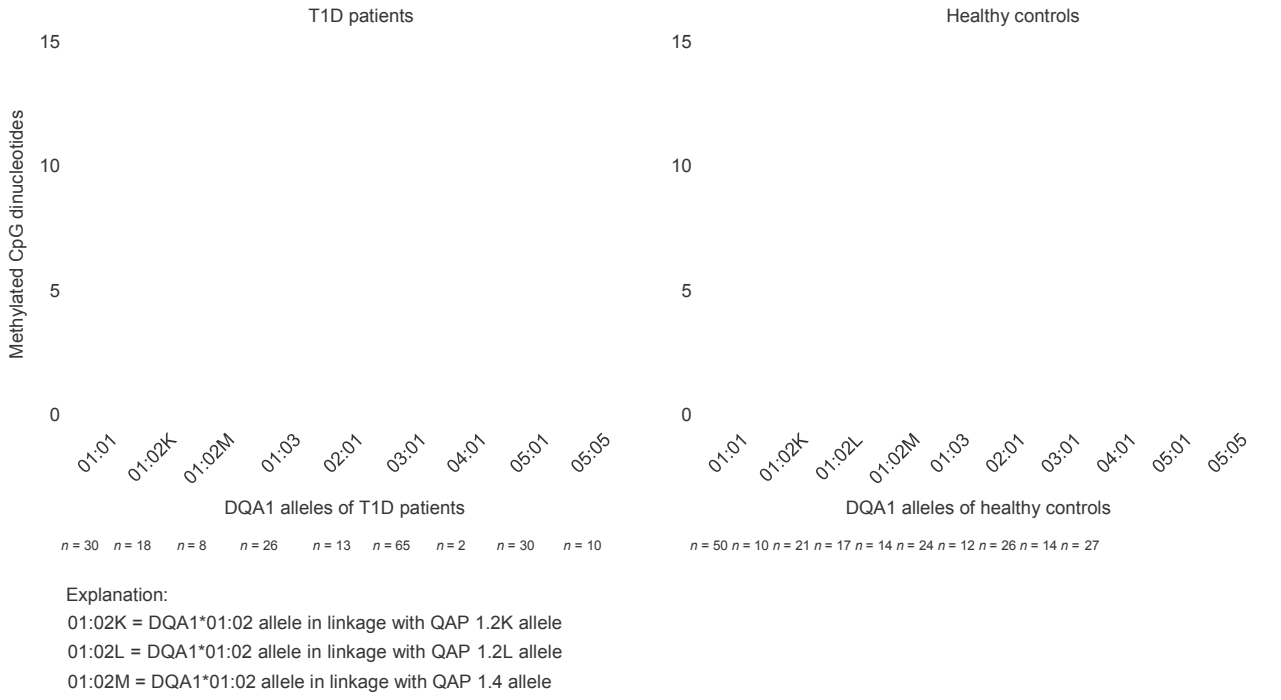


Figure 3. Total methylation profile of DQA1 promoter alleles in patients with type 1 diabetes mellitus (T1D) and healthy controls. Each point in the graph represents number of methylated CpG dinucleotides from single sequence of monocyte or whole blood leucocyte. Mean values with standard error of mean 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 ($\alpha = 0{\cdot}05$) followed by Bonferroni correction ($P_{corr} = P_{uncorr} * 10$).

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 pep- tide, but also on the stability of the resulting trimer. As we have performed whole blood leucocyte expression analysis, expression in more immunologically T1D-rele- vant cell populations (monocytes and B lymphocytes) should be assessed. Another possibility is that autoimmu- nity 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] Inter- estingly, 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

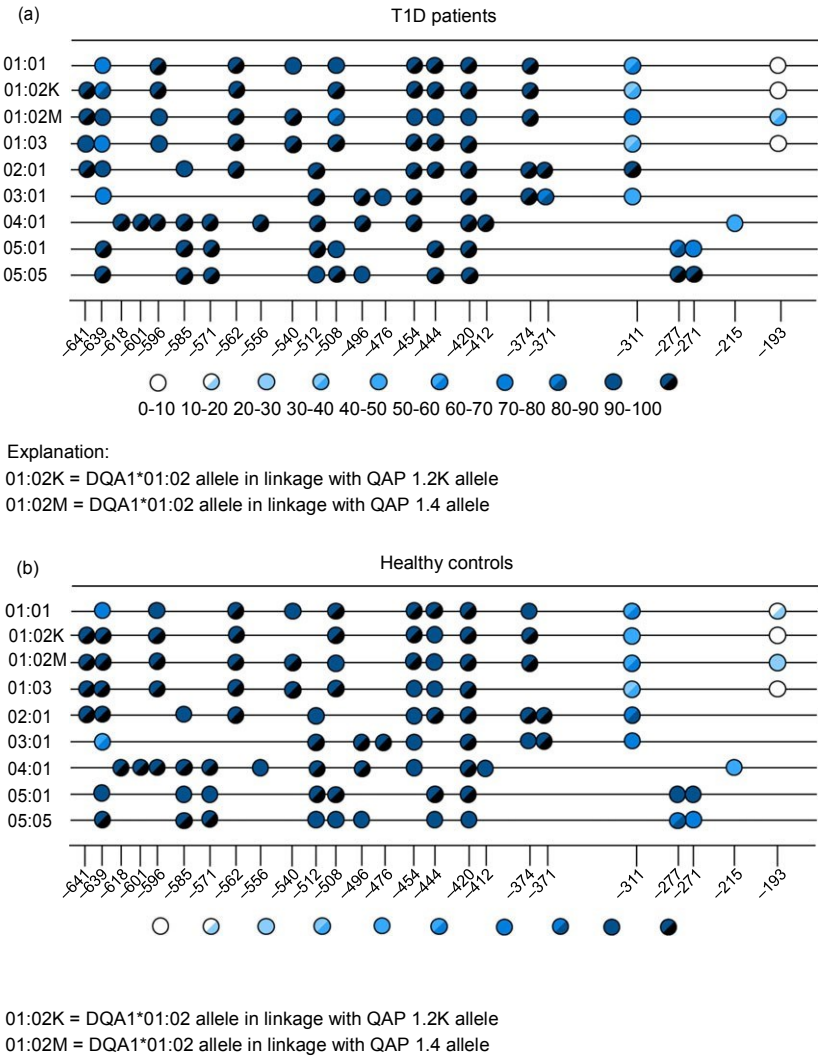


Figure 4. (a) Specific methylation profile of DQA1 promoter CpG sites in patients with type 1 diabetes mellitus (T1D). The matrix represents an amplicon that contains 9–12 CpG sites (number depends on promoter allele identity) obtained for 545 bp region overlapping promoter of the DQA1 gene. Each colour-coded 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 methyl- transferases 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 dif- ferent 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.

Acknowledgements

This survey was funded by the Research programme of Charles University in Prague: PRVOUK P31: Initial stadia of diabetes, obesity and other metabolic diseases. SVV/2016: Molecular, endocrine and genetic aspects of diabetes mellitus development. A.K.K. gratefully acknowledges the support of the Charles University through University Research Centre programme UNCE 204015.

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.

References

1 Gan MJ, Albanese-O'Neill A, Haller MJ. Type 1 diabetes: current concepts in epidemiology, pathophysiology, clinical care, and research. *Curr Probl Pediatr Adolesc Health Care* 2012; 42:269–91.

2 Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Erlich HA, *et al.* Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat Genet* 2009; 41:703–7.
3 Moustakas AK, Papadopoulos GK. Molecular properties of HLA-DQ alleles conferring susceptibility to or protection from insulin-dependent diabetes mellitus: keys to the fate of islet beta-cells. *Am J Med Genet* 2002; 115:37–47.
4 Cerna M. Genetics of autoimmune diabetes mellitus. *Wien Med Wochenschr* (1946) 2008; 158:2–12.
5 Swanberg M, Lidman O, Padyukov L, Eriksson P, Akesson E, Jagodic M, *et al.* MHC2TA is associated with differential MHC molecule expression and susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction. *Nat Genet* 2005; 37:486–94.
6 Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. *N Engl J Med* 2008; 359:2849–50.
7 Hyttinen V, Kaprio J, Kinnunen L, Koskenvuo M, Tuomilehto J. Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study. *Diabetes* 2003; 52:1052–5.
8 Fourlanos S, Varney MD, Tait BD, Morahan G, Honeyman MC, Colman PG, *et al.* The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes. *Diabetes Care* 2008; 31:1546–9.
9 Gillespie KM, Bain SC, Barnett AH, Bingley PJ, Christie MR, Gill GV, *et al.* The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. *Lancet* 2004; 364:1699–700.
10 Rakyan VK, Beyan H, Down TA, Hawa MI, Maslau S, Aden D, *et al.* Identification of type 1 diabetes-associated DNA methylation variable positions that precede disease diagnosis. *PLoS Genet* 2011; 7:e1002300.
11 Fradin D, Le Fur S, Mille C, Naoui N, Groves C, Zelenika D, *et al.* Association of the CpG methylation pattern of the proximal insulin gene promoter with type 1 diabetes. *PLoS One* 2012; 7:e36278.
12 Stefan M, Zhang W, Concepcion E, Yi Z, Tomer Y. DNA methylation profiles in type 1 diabetes twins point to strong epigenetic effects on etiology. *J Autoimmun* 2014; 50:33–7.
13 Kuroda A, Rauch TA, Todorov I, Ku HT, Al-Abdullah IH, Kandeel F, *et al.* Insulin gene expression is regulated by DNA methylation. *PLoS One* 2009; 4:e6953.
14 Morzycka-Wroblewska E, Harwood JI, Smith JR, Kagnoff MF. Structure and evolution of the promoter regions of the DQA genes. *Immunogenetics* 1993; 37:364–72.
15 Britten AC, Mijovic CH, Barnett AH, Kelly MA. Differential expression of HLA-DQ alleles in peripheral blood mononuclear cells: alleles associated with susceptibility to and protection from autoimmune type 1 diabetes. *Int J Immunogenet* 2009; 36:47–57.
16 Zajacova M, Kotrbova-Kozak A, Cepek P, Cerna M. Differences in promoter DNA methylation and mRNA expression of individual alleles of the HLA class II DQA1 gene. *Immunol Lett* 2015; 167:147–54.
17 Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15:539–53.
18 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16:1215.
19 Maffei A, Harris PE, Reed EF, Del Pozzo G, Ciullo M, Suciu-Foca N, *et al.* Differential expression of insulin-dependent diabetes mellitus-associated HLA-DQA1 alleles *in vivo*. *Eur J Immunol* 1997; 27:1549–56.
20 Donner H, Seidl C, Rau H, Herwig J, Seifried E, Usadel KH, *et al.* Unbalanced amounts of HLA-DQA1 allele mRNA: DQA1*03 shows high and DQA1*0501 low amounts of mRNA in heterozygous individuals. *Eur J Immunogenet* 2002; 29:321–30.
21 Fernandez S, Wassmuth R, Knerr I, Frank C, Haas JP. Relative quantification of HLA-DRA1 and -DQA1 expression by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). *Eur J Immunogenet* 2003; 30:141–8.
22 Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 2005; 5:793–806.
23 Ting JP, Trowsdale J. Genetic control of MHC class II expression. *Cell* 2002; 109 (Suppl):S21–33.
24 Miao F, Chen Z, Zhang L, Liu Z, Wu X, Yuan YC, *et al.* Profiles of epigenetic histone post-translational modifications at type 1 diabetes susceptible genes. *J Biol Chem* 2012; 287:16335–45.
25 DiMolfetto L, Neal HA, Wu A, Reilly C, Lo D. The density of the class II MHC T cell receptor ligand influences IFN-c/IL-4 ratios in immune responses *in vivo*. *Cell Immunol* 1998; 183:70–9.
26 Hermann R, Turpeinen H, Laine AP, Veijola R, Knip M, Simell O, *et al.* HLA DR-DQ-encoded genetic determinants of childhood-onset type 1 diabetes in Finland: an analysis of 622 nuclear families. *Tissue Antigens* 2003; 62:162–9.
27 Majumder P, Boss JM. DNA methylation dysregulates and silences the HLA-DQ locus by altering chromatin architecture. *Genes Immun* 2011; 12:291–9.
28 Scharer CD, Choi NM, Barwick BG, Majumder P, Lohsen S, Boss JM. Genome-wide CIITA-binding profile identifies sequence preferences that dictate function versus recruitment. *Nucleic Acids Res* 2015; 43:3128–42.

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.

Supplementary material

Table S1. DRB1–QAP–DQA1–DQB1 haplotypes in T1D patients and healthy controls. Occurrence rates of DRB1–QAP–DQA1–DQB1 haplotypes in patients suffering from T1D and in healthy controls are shown. DQA1*01:02 was found to be associated with 2 different promoters 1.2K and 1.4 depending on specific haplotype. Healthy controls were specifically selected to correspond with HLA frequencies of T1D patients. In total, 61 T1D patients and 39 healthy controls were included into the study. From 18 patients we have no expression data but only methylation profile of the HLA-DQA1 gene promoter. P values were calculated using chi square test (α=0.05) with Bonferroni correction $p_{corr} = p_{uncorr} * 10$.

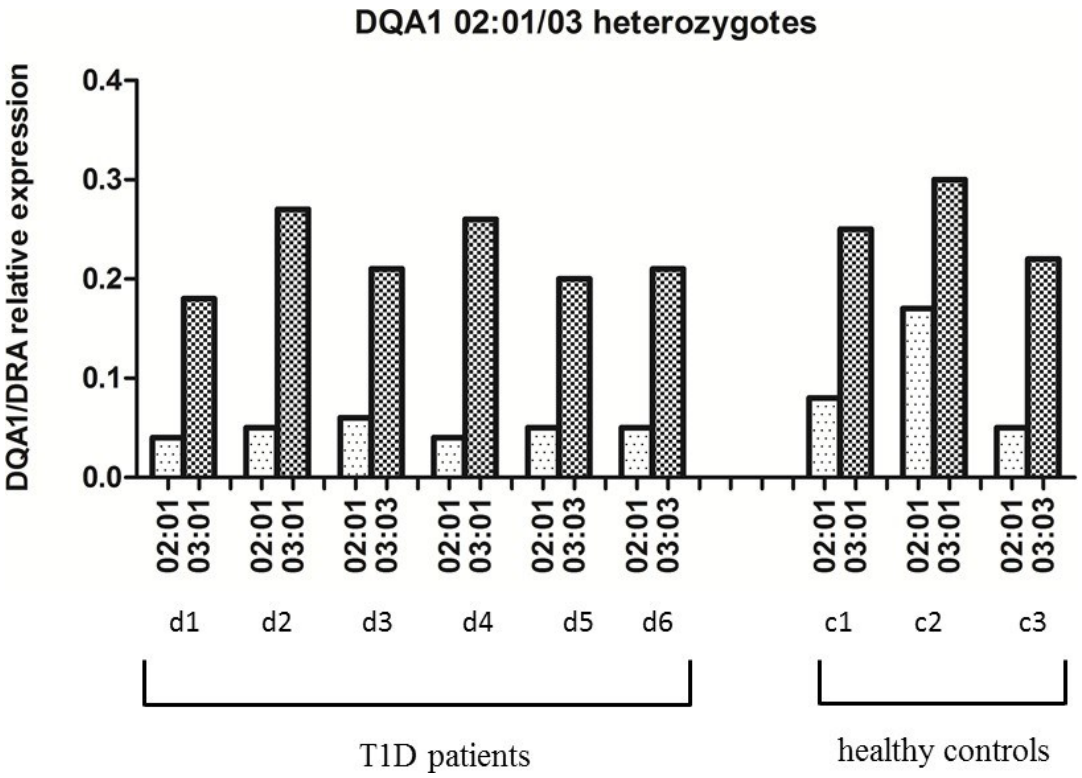
DRB1	QAP	DQA1	DQB1	T1D patients f (%)	Healthy controls f (%)	p_{corr}
04	3.1	03:01, 03:03	03:02	37.70	17.95	0.029
03	4.1B	05:01	02:01	21.31	15.38	1.000
01	1.1	01:01	05:01	11.48	10.26	1.000
07	2.1	02:01	02:02	9.02	19.23	0.362
13	1.3	01:03	06:03	4.10	7.69	0.652
11	4.1A	05:05	03:01	4.10	10.26	0.849
16	1.2K	01:02	05:02	3.28	1.28	1.000
08	4.2	04:01	04:02	1.64	2.56	1.000
04	3.1	03:03	03:01	1.64	1.28	1.000
04	3.1	03:01	03:05	0.82	1.28	1.000
13	1.4	01:02	06:04	3.28	--	
10	1.3	01:05	05:01	1.64	--	
15	1.2L	01:02	06:02	--	5.13	
14	1.3b	01:04	05:03	--	5.13	
07	2.1	02:01	03:03	--	1.28	
13	1.4	01:02	06:09	--	1.28	

f = haplotype frequency

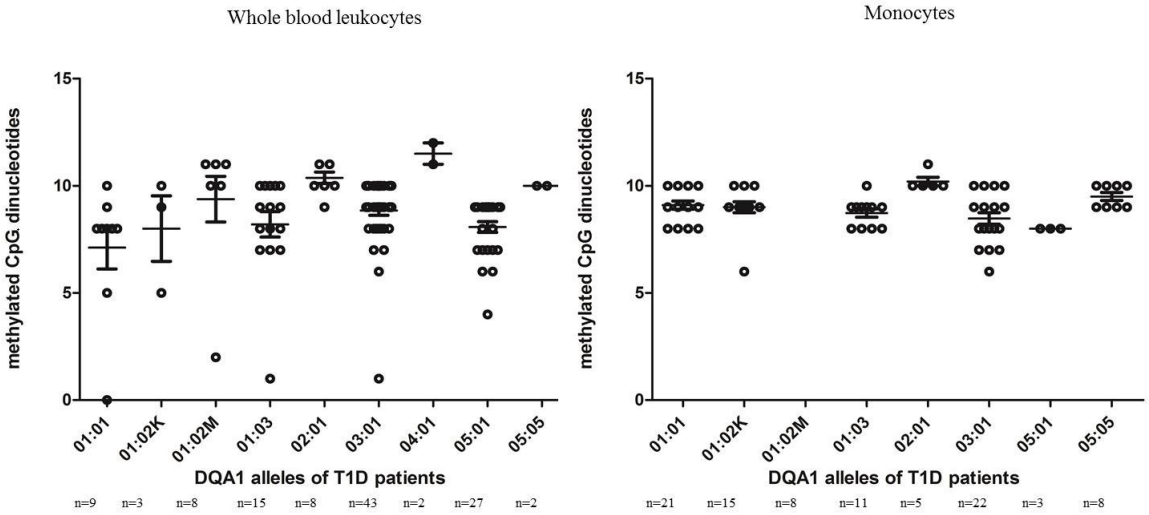
Table S2. Expression ratios of two alleles in DQA1 heterozygotes and their comparison between T1D patients and healthy controls.
Only T1D patients and healthy controls with at least 1 common haplotype are depicted. P values were calculated using Mann Whitney non parametric test (α=0.05) with Bonferroni correction ($p_{corr} = p_{uncorr} *2$).

DQA1 allele combination	QAP combination	n	Mean ratio allele 1/ allele 2	Range	SD	p_{corr}
03:01, 01:01	3.1, 1.1	5d	3.01	2.51-3.71	0.47	1.000
03:01, 01:01	3.1, 1.1	3c	3.05	2.47-3.47	0.52	
03:01, 01:02	3.1, 1.2K	1d	2.18	-	-	*
03:01, 01:02	3.1, 1.2K	4c	2.96	2.11-3.43	0.62	
03:01, 05:05	3.1, 4.1A	1d	4.02	-	-	*
03:01, 05:05	3.1, 4.1A	3c	4.51	4.33-4.67	0.17	
03:01, 01:03	3.1, 1.3	2d	2.02	1.90-2.13	0.16	*
03:01, 01:03	3.1, 1.3	2c	2.09	1.76-2.43	0.47	
03:01, 03:03; 02:01	3.1, 2.1	6d	4.87	3.45-6.68	1.19	0.190
03:01, 03:03; 02:01	3.1, 2.1	3c	2.96	1.72-4.16	1.22	
04:01, 02:01	4.2, 2.1	1d	1.06	-	-	*
04:01, 02:01	4.2, 2.1	2c	1.12	1.04-1.19	0.10	
05:01, 01:02	4.1B, 1.4	2d	1.86	1.47-2.25	0.55	*
05:01, 01:02	4.1B, 1.4	1c	1.64	-	-	
05:01, 02:01	4.1B, 2.1	2d	1.30	1.16-1.44	0.20	*
05:01, 02:01	4.1B, 2.1	3c	0.89	0.56-1.17	0.31	

n = number of individuals, SD = standard deviation, c = healthy controls, d = T1D patients
*Mann Whitney allows testing between two groups containing 3 or more values



S3. Relative expression of HLA DQA1*02:01/03 alleles in heterozygotes.
Two columns next to each other represent expression of two alleles in a DQA1*02**:**01/03 heterozygote. T1D patients show a lower expression of DQA1*02:01, particularly when it is in combination with DQA1*03. Mean allele ratio between these two alleles was in T1D patients (mean ratio 4.87) almost 2 times higher (not significant, $p_{corr} = 0.190$) than in healthy controls (mean ratio 2.96).



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

S4. Methylation profile of whole blood leukocytes and monocytes of T1D patients.
Each point in the graph represents the number of methylated CpG dinucleotides in a single sequence of monocyte or whole blood leukocyte. Mean values with standard error of the mean are indicated. Statistical significance was tested by non-parametric Kruskal-Wallis test with Dunn's posttest at the level of significance α=0.1. The number of analyzed sequences is indicated by n.

Annex 3

HLA-DRB1, -DQA1 and -DQB1 genotyping of 180 Czech individuals from the Czech Republic pop 3

Zajacova Marta, Kotrbova-Kozak Anna, Cerna Marie

Human Immunology 77 (2016) 365–366

Human Immunology 77 (2016) 365–366

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

HLA-DRB1, -DQA1 and -DQB1 genotyping of 180 Czech individuals from the Czech Republic pop 3

Zajacova Marta, Kotrbova-Kozak Anna, Cerna Marie ⇑

Dept. of General Biology and Genetics, Third Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

articleinfo

Article history:
Received 2 December 2015
Revised 4 February 2016
Accepted 4 February 2016
Available online 8 February 2016

Keywords:
HLA alleles
DRB1
DQB1
DQA1
Czech population

abstract

One hundred and eighty Czech individuals from the Czech Republic pop 3 were genotyped at the HLA-DRB1, -DQA1 and -DQB1 loci using sequence-specific primers PCR methods. HLA-DRB1, -DQA1 and -DQB1 genotypes are consistent with expected Hardy–Weinberg (HW) proportions. These genotype data are available in the Allele Frequencies Net Database under identifier AFND.

The Czech Republic, the state in the Middle Europe, comprises three historical countries – Bohemia (west part), Moravia (east part) and Silesia (small part to the north of Moravia, majority of Silesia is included in Poland). As of 2015, the population of the Czech Republic numbered approximately 10.5 million. About 96% of the population is Czech. More than half of foreigners (originated from Slovakia, Ukraine, Poland, Vietnam, Germany, Russia and Hungary) live in the capital, Prague, and Middle Bohemia. The population of Prague itself numbers 1.2 million; the population of the metropolitan region of Prague numbers 2.3 million, so which is more than one fifth of total population of the Czech Republic. The Czech population is descended from the indigenous Slavic population, which had reached this region in the 5th and the 6th centuries, with historical admixture of the Celtic and German populations from this period. The German immigration has continued throughout all the Middle Ages and modern times. The main spoken language is Czech.

In order to investigate the HLA diversity of the Czech population, DNA of one hundred and eighty unrelated Czech healthy volunteers living in Prague was collected. These 180 subjects were recruited from students and employees of Charles University in Prague. Charles University students and employees usually originate from all parts of the Czech Republic. Thus, these individuals should be considered a representative sample of the general population. Informed consent was obtained for the donation and use of genotype data for these anonymized individuals for research and public dissemination.

HLA-DRB1, -DQA1 and -DQB1 genotypes were obtained by polymerase chain reaction with sequence-specific primers (SSP-PCR) using Olerup SSP™ typing kits (Olerup SSP AB, Stockholm, Sweden). IMGT/HLA database release 3.10.0. was used. HLA-DQA1 was genotyped using Olerup SSP™ HLA-DQA1 typing kits. 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 [1,2].

Allele and haplotype frequencies were determined via direct counting. HWE was assessed by chi square test using Prism 3.0 software. No deviations from Hardy–Weinberg equilibrium proportions (HWEP) were detected for neither HLA-DRB1-DQA1-DQB1 haplotypes, nor for individual loci. These genotype data are available in the Allele Frequencies Net Database (AFND) [3] under the population name "Czech".

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.humimm.2016.02.003.

⇑ Corresponding author.
E-mail address: marie.cerna@lf3.cuni.cz (M. Cerna).

http://dx.doi.org/10.1016/j.humimm.2016.02.003
0198-8859/

References

[1] M. Fojtikova et al., HLA class II, MICA and PRL gene polymorphisms: the common contribution to the systemic lupus erythematosus development in Czech population, Rheumatol. Int. 31 (2011) 1195–1201.

[2] M. Zajacova et al., Differences in promoter DNA methylation and mRNA expression of individual alleles of the HLA class II DQA1 gene, Immunol. Lett. 167 (2015) 147–154.

[3] F.F. Gonzalez-Galarza, L.Y.C. Takeshita, E.J.M. Santos, F. Kempson, M.H.T. Maia, A.L.S. da Silva, et al., Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations, Nucleic Acids Res. 43 (2015) D784–D788, http://dx.doi.org/10.1093/nar/gku1166.

Annex 4

Expression of HLA-DQA1 and HLA-DQB1 genes in B lymphocytes, monocytes and whole blood

Marta Zajacova, Anna Kotrbova-Kozak, Marie Cerna

International Journal of Immunogenetics (2018) In Press

Received: 15 May 2017 | Revised: 23 February 2018 | Accepted: 22 March 2018

DOI: 10.1111/iji.12367

ORIGINAL ARTICLE

WILEY INTERNATIONAL JOURNAL OF IMMUNOGENETICS

Expression of HLA-DQA1 and HLA-DQB1 genes in B lymphocytes, monocytes and whole blood

M. Zajacova | A. Kotrbova-Kozak | M. Cerna

Department of Medical Genetics, Third Faculty of Medicine, Charles University, Prague, Czech Republic

Correspondence
Marie Cerna, Department of Medical Genetics, Third Faculty of Medicine, Charles University, Prague, Czech Republic.
Email: marie.cerna@lf3.cuni.cz

Funding information
This survey was funded by the research programme of Charles University: PROGRES Q36 Metabolism 260387/SVV/2018: Molecular, endocrine and genetic aspects of diabetes mellitus aetiology. AKK gratefully acknowledges the support of the Charles University through University Research Centre programme UNCE 204015.

Summary

Differential expression of HLA-DQA1 and HLA-DQB1 gene alleles was analysed in three different cell populations isolated from peripheral blood—B lymphocytes, monocytes and whole-blood cells. Interallelic differences in mRNA levels were observed: DQA1*03 alleles were among the most expressed in all cell types, whereas DQA1*05 alleles were least expressed in whole blood and monocytes and among the most expressed in B cells. For DQB1 gene, DQB1*06 group of alleles were the most expressed, and DQB1*02 group the least expressed within all cell populations examined. In comparison with the rest alleles, DQB1*06 and DQB1*05:02 alleles have higher expression in monocytes than in B cells, professional antigen-presenting cells. Cell type-specific regulation of expression was observed as well, with higher and more balanced expression of alleles in B lymphocytes compared to monocytes.

KEYWORDS
antigen presentation, gene expression, genetic polymorphism, HLA class II, HLA-DQA1, HLA-DQB1

1 | INTRODUCTION

Human leucocyte antigen (HLA) class II molecules play a central role in the adaptive 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 crucial for the initiation of the adaptive immune response (Reith, LeibundGut-Landmann, & Waldburger, 2005). 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 (Cerna, 2008).

The expression of HLA class II is regulated mainly on the transcriptional level. Proximal promoters of all class II genes contain conserved regulatory sequences—W (or S), X and Y boxes—which are crucial for the expression. Sequences of S-X-Y module are recognized and bound by ubiquitous, constitutively expressed protein factors; however, the resulting multiprotein enhanceosome is not sufficient to activate the transcription by itself. Instead, it serves as a platform for binding of major class II transcriptional regulator, class II transactivator (CIITA; Reith et al., 2005).

CIITA controls transcription of all HLA class II genes very tightly and in a coordinated fashion (Otten & Steimle, 1998; Reith et al., 2005). Although some exceptions exist (Douhan, Lieberson, Knoll, Zhou, & Glimcher, 1997), it can be generally said that without CIITA, there is no class II transcription, and there is strong positive correlation between levels of the CIITA protein or its corresponding mRNA in the cell and the level of any of the HLA class II transcripts (Cazalis et al., 2013; Otten & Steimle, 1998). In addition, essentially all inflammatory stimuli that boost HLA class II expression act through CIITA (Ting, Trowsdale, Hill, Carolina, & Allcock, 2002).

HLA class II genes display high level of polymorphism (with the exception of HLA-DRA), with variants ranging from tens to several hundreds 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 (Cerna, 2008). However, sequence differences are also abundant within the promoters of DRB1 (Perfetto, Zacheis, McDaid, Meador, & Schwartz, 1993), DQA1 (Del Pozzo et al., 1992; Morzycka-Wroblewska,

IJI | Journal Name
12367 | Manuscript No.
WILEY
Dispatch: 6-4-2018 | No. of pages: 10
CE: Arockia Raj P | PE: Saranya Shree

Harwood, Smith, & Kagnoff, 1993) and DQB1 (Andersen et al., 1991) genes. Promoter polymorphism is functional and translates into variable levels of expression of HLA-DQA1 (Britten, Mijovic, Barnett, & Kelly, 2009; Donner et al., 2002; Fernandez, Wassmuth, Knerr, Frank, & Haas, 2003; Morzycka-Wroblewska, Munshi, Ostermayer, Harwood, & Kagnoff, 1997), DQB1 (Britten et al., 2009) and DRB1 (Vincent et al., 1996) alleles.

For binding of CIITA to S-X-Y module, besides intact S, X and Y boxes with their protein partners, their exact stereospecific alignment is needed (Harton & Ting, 2000). Any modification to either the sequence of these binding sites or their respective distances can affect ability of HLA promoter to drive the expression of its gene. Polymorphisms have been described in Y box that modifies its ability to bind its cognate protein NF-Y, which could account for variation in promoter strength between DQA1 alleles and also between DQA1 and DRA genes (Indovina et al., 1998). Similarly, X box-binding factor RF-X shows gradient of affinities for the X boxes of DRA, DPA and DQA genes (Kobr, Reith, Herrero-Sanchez, & Mach, 1990).

It is tempting to speculate that these allele- and cell type-specific mechanisms interact and that HLA class II alleles are subject to different types of regulation in different cell types.

Previously, we have described differences in mRNA expression of individual HLA-DQA1 alleles in unsorted cells from whole blood (Zajacova, Kotrbova-Kozak, Cepek, & Cerna, 2015). In this study, we aimed at analysing expression of HLA-DQA1 and HLA-DQB1 gene alleles not only in whole blood, but also in specific cell types—B lymphocytes (as "professional" APCs) and monocytes, which are less efficient APCs (Laupeze et al., 1999).

2 | MATERIAL S AND METHODS

2.1 | Subject of the study

The test group consisted of 42 healthy volunteers of European descent, 19–69 years of age (mean 31.2 ± 12.1).

2.2 | DQA1, DRB1 and DQB1 genotyping

Genomic DNA from peripheral leucocytes was extracted using salt extraction (ethanol precipitation) method (modified protocol of Miller, Dykes, & Polesky (1988)), and DQA1, DQB1 and DRB1 genotyping was performed using Olerup SSP typing kits as described previously (Zajacova et al., 2015).

2.3 | Isolation of cell populations, RNA extraction, cDNA synthesis and quantification

CD19+ B lymphocytes and CD14+ monocytes were isolated from peripheral blood using Dynabeads® CD19 Pan B and Dynabeads® CD14 (Invitrogen, Waltham, MA, USA) magnetic beads. Total RNA was extracted from whole blood using QIAamp RNA Blood Mini Kit (Qiagen, Venlo, the Netherlands). RNA from purified cell populations was isolated by total RNA purification kit (Sigma-Aldrich, St. Louis, MI, USA). RNA was reverse-transcribed with random hexamer primers using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) and quantified on 7500 Fast Real-Time PCR System (Applied Biosystems) as described before (Zajacova et al., 2015). Amplification curves were visually inspected, and reactions whose slopes strongly differed from the slopes of the same-assay reactions on the same plate were discarded. Values obtained for allele-specific assays were corrected according to the assay efficacy and subsequently according to the genomic DNA content of the sample to reflect proportions in the RNA component of the sample only.

2.4 | Primers and probes for expression analysis

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, *05 and DQB1*02, *03:01, *03x amplify alleles *03:02–03:05 and *04, DQB1*05, *06 assays). "DQA1 total" and "DQB1 total" assays were used to quantify total DQA1 and DQB1 mRNAs, respectively, irrespective of the alleles present, "DRA" assay targeting the HLA-DRA gene was used as an endogenous control, and assays amplifying a nonpolymorphic intronic segment of the DQA1 gene "DQA1 intron" and DQB1 gene "DQB1 intron" were used to determine the genomic DNA contamination in the samples. The design of DQA1 and DRA PCR primers and fluorogenic probes (synthetized by IDT, Coralville, IA, USA) was based on primers and probes used by Fernandez et al. (2003) and modified according to published sequences (http://www.ebi.ac.uk/ipd/imgt/hla/align.html). As DQA1 assay was found to target DQA2 gene as well, and DQA2 gene was reported to be expressed in some cell types (Lenormand et al., 2012; Yu & Sheehy, 1991), an additional assay "DQA2" was designed to quantify amplification of this gene. Expression of DQA2 gene was found to be less than 0.01% of DQA1 gene expression (i.e. at least 14-cycle difference) in all samples. Sequences and properties of all primers and probes are listed in Table S1.

2.5 | Detection of genomic DNA contamination, assay specificity and assessment of relative amplification efficiencies

The content of genomic DNA in the samples was determined using assays amplifying nonpolymorphic intronic segment of the DQA1 gene and DQB1 gene (Fernandez et al., 2003). Genomic DNA contamination was found to be 0%–19% (mean 4.7 ± 3.8%) for DQA1 gene and 0%–32% (mean 9.8 ± 7.3%) for DQB1 gene. We were unable to identify a reason for discrepancy between assessment of DNA content by DQA1 and DQB1 intronic assay.

Specificity of each allele-specific assay was verified using cDNA of individuals carrying off-target allelic groups as a template in amplification reaction. The proportion of the off-target amplicons was found to be less than 0.02% of the target allele's amplicons (i.e. at least 12-cycle difference) for all assays used.

To determine the efficacy of allele-specific assays, DQB1 total assay was assigned efficacy $E_{totalB} = 1$, and mean C_t of DQB1 total assay was compared with mean Cts of DQB1*02, *0301, *03x, *05 and *06 in all available individuals homozygous for a given DQB1 allele. Then, for each assay, the assay efficacy was calculated from the data of individuals heterozygous for this allele and also for the allele with already assessed efficacy. The assay efficacy was determined as an average of values obtained from "homozygote" and "heterozygote" calculations. If no homozygote for given allele was present in a sample, we used only "heterozygote" data as efficacy of the assay.

Relative efficacies of "DQB1 total" (E_{totalB}), "DQB1 intron," "DQA1 total" and "DQA1 intron" were determined using 2^0 to 2^{-7} serial dilutions of input cDNA as a template for amplification in all four assays. Standard curves were constructed, E_{totalB} was assigned a value of 1, and efficacy of the three assays relative to E_{totalB} was assessed by comparing the slopes. Then, efficacy of DQA1 allele-specific assays was determined by comparing mean C_t of DQA1 total assay with mean Cts of allele-specific assays in a same way as it was made for DQB1 gene.

E_{DRA} was previously found to be similar to E_{totalA}; therefore, it was also assigned a value of 1 (Zajacova et al., 2015). PPIA assay (Applied Biosystems) efficacy was determined using 10^0 to 10^{-4} serial dilutions of input cDNA as a template for amplification in both PPIA and DQA1 total assays and by comparing the standard curve slopes for both assays. Efficacy of PPIA assay was 1.001.

2.6 | Analysis of cell type-specific and allele-specific mRNA expression

Relative expression of DQA1 and DQB1 alleles was determined using the amount of HLA-DRA mRNA as the reference. To ensure the reliability of results, only samples where (allele 1+ allele 2 mRNA)/(total DQA1 or DQB1 mRNA) was in the <0.84; 1.16> interval were included in the subsequent analyses, as described previously (Zajacova et al., 2015). In homozygous individuals, both copies of DQA1 or DQB1 gene were considered to be transcribed equally; therefore, the detected allele mRNA level in homozygotes represented double the amount of mRNA from a gene copy and was divided by 2.

To assess general differences in allele expression between cell populations, all alleles were analysed together in bulk. As all measurements did not pass the quality criterion described above, for several individuals we obtained measurements in only one or two of three studied cell populations. Therefore, to avoid skewing of results by overrepresentation of a specific allele in a cell type, only paired values (measurements of allele expression in whole blood, B lymphocytes and monocytes of the same individual) for each combination of cell types were used. Differences in expression of all DQA1, and then DQB1, alleles between cell types were tested by two-tailed Wilcoxon's matched-pairs test with 95% CI. After comparison of all possible combinations of cell types, we compensated for multiple comparisons using the Bonferroni correction (three groups tested, $p_{corrected(c)} = p_{uncorrected(un)} \times 3$).

Differences in the expression of specific DQA1 and DQB1 alleles between cell types were tested by two-tailed Mann–Whitney test with 95% CI. Expression of each allele was compared between each of the two cell types, and the compensation for multiple comparisons was made by Bonferroni correction (nine alleles tested, $p_c = p_{un}*9$ for both DQA1 and DQB1).

Differences in the expression of individual alleles within each cell type were assessed by two-tailed Mann–Whitney test with 95% CI. All p-values were subjected to the Bonferroni correction for multiple comparisons (nine alleles tested, $p_c = p_{un}*9$).

To obtain a statistically significant result using the method described above, a minimum of 4 + 8 or 5 + 6 samples in the compared groups is needed.

Of 165 comparisons for DQA1 gene, 37 contained enough samples to give significant result, and among them, 22 proved differences between tested groups. Of 273 possible comparisons for DQB1 gene, differences could possibly be found in 31 and were found in 17.

Expression of DRA in cell types was compared by two-tailed Mann–Whitney test with 95% CI, and p-values were subjected to the Bonferroni correction ($p_c = p_{un}*3$). Correlation between C_t of DRA and C_t of PPIA gene was analysed by Spearman's rank correlation coefficient and Pearson's product–moment correlation coefficient. Linear regression was used to quantitatively describe relationship between these two values.

3 | RESULTS

The transcription rates in 42 individuals were analysed. Data included 10 HLA-DQA1 alleles and 13 DQB1 alleles. As allele DQA1*01:02 is linked to three different DQA1 promoters in three different haplotypes, we treated it as three different alleles (DR13-, DR15- and DR16-linked DQA1*01:02 allele).

3.1 | Relative expression of HLA class II alleles in different cell types

First, we analysed differences in DQA1 and DQB1 gene allele expression between cell types, irrespective of allele identity. We observed significant difference in relative DQA1 allele expression between all cell types tested—B lymphocytes and monocytes, B lymphocytes and whole-blood cells, and monocytes and whole blood (all $p_c < .0003$). The highest DQA1 level was observed in B lymphocytes, whole-blood cells expressed around half of the amount observed in B lymphocytes, and monocytes expressed around one-third of B cell levels (Figure 1a).

For DQB1 gene alleles, the only significant difference was observed between B lymphocytes and monocytes ($p_c = .0213$), where DQB1 allele expression was on average 1.25 times higher in B cells (Figure 1b).

Then, we examined differences in expression between cell types on the level of individual alleles (Figure 2). For DQA1 gene, mean

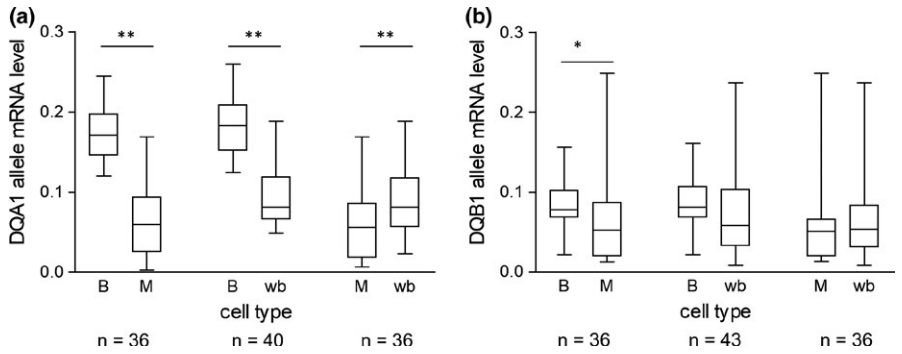


FIGURE 1 Relative mRNA expression of DQA1 (a) and DQB1 (b) alleles in different cell types. Normalized expression data of all DQA1 (a) and DQB1 (b) alleles in the indicated cell type were grouped together irrespective of allele identity, and differences in allele expression between cell types were analysed by two-tailed Wilcoxon's matched-pairs test. Only individuals for whom measurements in both compared cell types were available (paired measurements) were included in the analysis. The number of samples analysed (n) and cell type (wb—whole-blood cells, B—B lymphocytes, M—monocytes) is indicated. *$p_c < .05$, **$p_c < .005$

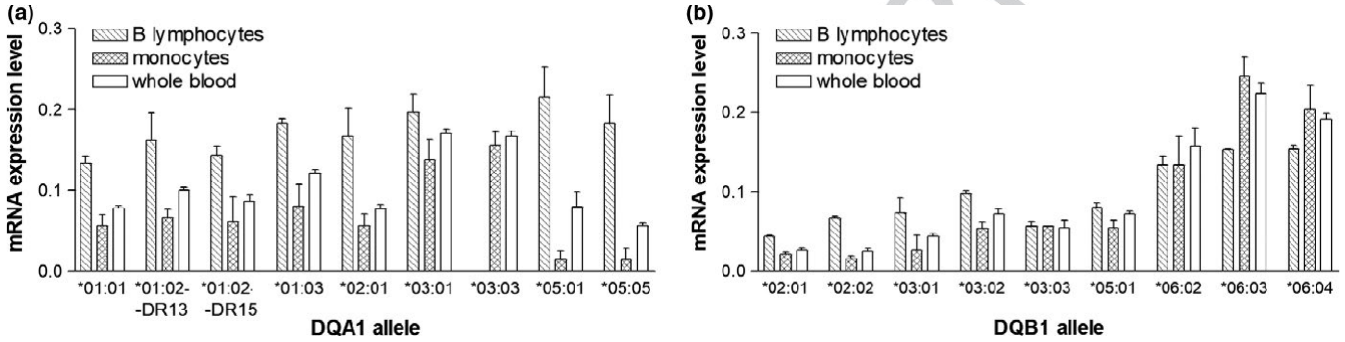


FIGURE 2 mRNA expression levels of DQA1 (a) and DQB1 (b) alleles in different cell types. Expression of DQA1 (a) and DQB1 (b) alleles was determined by qPCR with allele-specific primers, and the mean of three independent measurements was used for the analysis. Each column represents mean of data of at least two individuals, and SD is indicated

relative expression level followed the same pattern in each allele without exception—highest in B lymphocytes and lowest in monocytes. Difference between these two cell populations varied from expression being 1.3 times higher in B lymphocytes for DQA1*03 group alleles to more than 13 times difference in the transcription of DQA1*05 alleles. Expression in whole blood was intermediate, with transcript levels lying between the values for both cell populations. Due to the low number of samples in some allelic groups, we were able to statistically compare expression of DQA1 *01:01, *02:01, *03:01 and *05:05 alleles only. For DQA1*02:01,*03:01 and *05:05 alleles, differences in expression between B cells and monocytes proved to be significant, as well as differences between B cells and whole blood for alleles DQA1*02:01 and *05:05.

The situation was not so unambiguous for the DQB1 gene. For most of DQB1*02 and *03 group alleles and for DQB1*05:01 allele, expression pattern correlated with that of DQA1 gene, with the highest mean allele expression in B lymphocytes and lowest in monocytes. However, for DQB1*06 allele group, this seemed to be reversed and the lowest relative expression of allele was observed in B lymphocytes. The expression of DQB1*03:03 and *05 alleles in B lymphocytes and monocytes seemed to be similar. Generally, difference in expression between B cells and monocytes was lower than that for DQA1 gene, with a maximum 4 times higher expression in B cells observed for DQB1*02:02 allele.

We were able to statistically test differences between cell types for alleles DQB1*02:02, *03:01 and *05:01. The expression of DQB1*02:02 and *03:01 alleles was significantly higher in B lymphocytes compared to monocytes and compared to whole blood.

Means of allelic mRNAs and ratios of mean allelic mRNA expression in all cell types are listed in Table 1.

3.2 | Interallelic differences in expression of DQA1 and DQB1 genes

Next, we inspected the differences in relative expression between individual alleles. In DQB1 gene, alleles of *02, *03 and *05 groups were less expressed than *06 group alleles, and this relationship was observed in all cell types examined. In monocytes, the average mRNA level of the most expressed allele DQB1*06:03 was up to 16 times higher than that of the least expressed *02:02 allele. In B cells, the expression of individual alleles was more balanced than in monocytes; the most highly expressed allele DQB1*06:04 showed 3.5 times higher average transcription rate than the least expressed *02:01 (Figure 2, Table 2).

TABLE 1 Comparison of mRNA expression of DQA1 (a) and DQB1 (b) alleles in different cell types

	Relative expression of allele in cell type			Expression ratio in different cell types			Number of samples
	B	M	wb	B/M	B/wb	wb/M	B; M; wb
(a)							
DQA1*01:01	0.133	0.056	0.078	2.36	1.70	1.39	4; 5; 6
DQA1*01:02 (DR13 linked)	0.162	0.066	0.100	2.44	1.62	1.51	3; 2; 4
DQA1*01:02 (DR15 linked)	0.144	0.061	0.087	2.35	1.66	1.42	4; 3; 3
DQA1*01:02 (DR16 linked)	0.146	0.077	0.100	1.90	1.46	1.30	1; 1; 1
DQA1*01:03	0.183	0.080	0.121	2.62	1.52	1.72	4; 4; 5
DQA1*02:01	0.167	0.056	0.077	2.96[a]	2.18	1.36[a]	5; 7; 7
DQA1*03:01	0.196	0.137	0.170	1.43[b]	1.15	1.24	8; 7; 5
DQA1*03:03	0.205	0.156	0.167	1.32	1.23	1.07	1; 3; 2
DQA1*05:01	0.215	0.015	0.065	14.82	2.69	5.50	5; 4; 5
DQA1*05:05	0.183	0.015	0.056	13.10[a]	3.25	4.03[b]	12; 11; 13
Max/min	1.6	10.4	3.0				
(b)							
DQB1*02:01	0.044	0.022	0.027	2.00	1.63	1.23	4; 4; 6
DQB1*02:02	0.067	0.016	0.025	4.11[1]	2.69[2]	1.52	6; 6; 7
DQB1*03:01	0.073	0.026	0.044	2.77[2]	1.67[1]	1.66	10; 8; 12
DQB1*03:02	0.098	0.054	0.072	1.82	1.35	1.34	5; 4; 5
DQB1*03:03	0.057	0.056	0.054	1.01	1.04	0.96	2; 2; 2
DQB1*03:05	0.075	0.050	0.014	1.50	5.43	0.28	1; 1; 1
DQB1*05:01	0.081	0.054	0.072	1.52	1.14	1.33	5; 6; 5
DQB1*05:02	0.109	0.111	0.116	0.98	0.94	1.04	1; 1; 1
DQB1*05:05	–	0.051	0.057	–	–	1.13	0; 1; 1
DQB1*06:02	0.134	0.134	0.158	1.00	0.85	1.19	5; 4; 4
DQB1*06:03	0.154	0.246	0.224	0.62	0.69	0.91	2; 3; 2
DQB1*06:04	0.155	0.204	0.192	0.76	0.81	0.94	3; 2; 3
DQB1*06:09	0.143	–	0.178	–	0.80	–	1; 0; 1

Expression hierarchy of DQA1 gene seemed to follow the same pattern in monocytes and in whole blood, with the lowest expression levels in DQA1*05:05 and *05:01 alleles, gradually increasing through *02:01 and *01:01 alleles, *01:02 and *01:03 alleles, and expression peaking in DQA1*03 alleles (three times higher than in *05 allele group). In B lymphocytes, similar to monocytes, DQA1 *01:01, *01:02 (DR15) and *02:01 belonged to alleles with low expression and *03 group alleles belonged to the higher part of expression spectrum. However, it was surprising to see that DQA1*05 alleles, whose expression level in monocytes was the lowest of all alleles, were among the most expressed alleles in B lymphocytes, with an expression level similar to that of *03 allele group. Similar to DQB1 gene, expression of alleles was more balanced in B lymphocytes (mean mRNA level of the most expressed allele DQA1*05:01 was 1.6 times higher than that of the least expressed allele DQA1*01:01) than in monocytes (most expressed allele DQA1*03:03 was on average expressed 10 times more than *05:05).

3.3 | Correlation between PPIA and DRA gene expression

To analyse the strength of HLA class II promoting stimuli in each cell type, the expression of DRA gene normalized against classic

TABLE 2 Expression hierarchy of individual alleles of DQA1 (a) and DQB1 (b) genes

	Mean DQA1 allele expression	Statistically significant results	Most/least expressed allele ratio	Relative expression of the most expressed allele
(a)				
B	01:01 < 01:02 (DR15) < 01:02 (DR13) < 02:01 < 05:05 = 01:03 < 03:01 < 05:01	01:01, 01:02 (DR15) < 03:01	1.62	0.215
M	05:05 = 05:01 < 02:01 < 01:01 < 01:02 (DR15) < 01:02 (DR13) < 01:03 < 03:01 < 03:03	05:05 < 01:01, 01:03, 02:01, 03:01; 0101, 02:01 < 03:01	10.4	0.156
wb	05:05 < 05:01 < 02:01 = 01:01 < 01:02 (DR15) < 01:02 (DR13) < 01:03 < 03:03 < 03:01	05:05 < 01:01, 01:02, (DR13), 01:03, 03:01; 01:01, 02:01 < 01:03, 03:01	3.04	0.170
(b)				
B	02:01 < 03:03 < 02:02 < 03:01 < 05:01 < 03:02 < 06:02 < 06:03 < 06:04	–	3.52	0.155
M	02:02 < 02:01 < 03:01 < 03:02 = 05:01 < 03:03 < 05:02 < 06:02 < 06:04 < 06:03	02:02, 03:01 < 06:02; 02:02 < 03:02, 05:01	15.38	0.246
wb	02:02 < 02:01 < 03:01 < 03:03 < 03:02 = 05:01 < 05:02 < 06:02 < 06:04 < 06:03	02:01, 02:02 < 03:01, 03:02, 05:01; 03:01 < 03:02, 05:01, 06:02	8.96	0.224

Expression data were normalized relative to DRA gene, and mean relative expression was calculated for each combination of allele and cell type. Only alleles with measurements from at least two individuals are listed. Then, alleles were sorted according to expression means, and mRNA ratio of the most and the least expressed alleles in each cell type was calculated. B lymphocytes (B), monocytes (M), whole blood (wb).

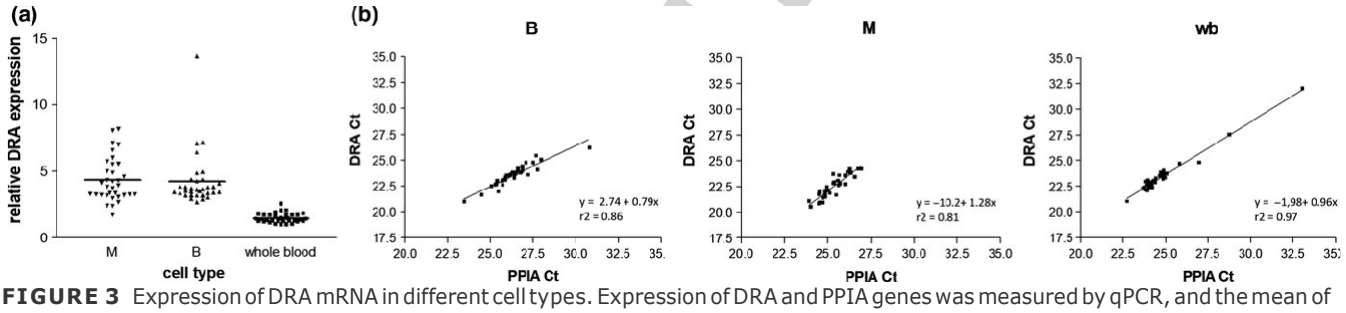


FIGURE 3 Expression of DRA mRNA in different cell types. Expression of DRA and PPIA genes was measured by qPCR, and the mean of three independent measurements was used for the analysis. (a) DRA mRNA levels normalized against PPIA in B lymphocytes (B), monocytes (M) and whole blood (wb). (b) Correlation between PPIA and DRA mRNA expression in different cell types. C_t values of DRA gene assay are plotted against C_t values of PPIA assay. Linear regression curves and r^2 values are shown for each curve

endogenous control PPIA was analysed. Expression was found to be significantly more pronounced in B lymphocytes (mean relative DRA expression 4.2) and monocytes (4.9) compared to whole blood (1.9; both $p_c < .0003$; Figure 3a). There was significant monotonic and linear correlation between C_t of DRA and C_t of PPIA in each cell type (Figure 3b). The value of Spearman's correlation coefficient was 0.88 in whole blood, 0.94 in B lymphocytes and 0.89 in monocytes, and Pearson's correlation coefficient was 0.99 in whole blood, 0.93 in B lymphocytes and 0.90 in monocytes (all six p values < .0001). Linear correlation between Cts means that ratio of PPIA to DRA in individual cell types tends to be constant among individuals. However, the slopes and constants of the equations describing this linear relationship were different for each cell type, indicating a cell type-specific relationship between the amount of DRA and PPIA transcripts.

3.4 | Relative expression of DQA-DQB haplotypes in different cell types

Last, we compared the relative expression of DQA1 and DQB1 genes in the haplotypes with respect to the B lymphocyte/monocyte ratio. We might divide the haplotypes into three groups according to the relation of B/M between DQA1 and DQB1 genes. The first group includes DR4 and DR7 haplotypes (DQA1*03:01–DQB1*03:01, DQA1*03:01–DQB1*03:02, DQA1*03:01–DQB1*03:05, DQA1*03:03–DQB1*02:02, and DQA1*02:01–DQB1*02:02), where both genes have higher expression in B lymphocytes than monocytes, and the difference is more pronounced for DQB1 gene (up to three times more for DQA1*03:03–DQB1*02:02). The second group includes DR1 (DQA1*01:01–DQB1*05:01), DR3 (DQA1*05:01–DQB1*02:01),

TABLE 3 Relative expression of DQA1 and DQB1 genes in the haplotypes with respect to the B lymphocyte/monocyte ratio

DQA1–DQB1 haplotype	Expression ratio of B/M for DQA1	Expression ratio of B/M for DQB1
DQA1*01:01–DQB1*05:01	2.36	1.52
DR13-DQA1*01:02–DQB1*06:04	2.44	0.76
DR15-DQA1*01:02–DQB1*06:02	2.35	1.00
DR16-DQA1*01:02–DQB1*05:02	1.90	0.98
DQA1*01:03–DQB1*06:03	2.62	0.62
DQA1*02:01–DQB1*02:02	2.96	4.11
DQA1*02:01–DQB1*03:03	2.96	1.01
DQA1*03:01–DQB1*03:01	1.43	2.77
DQA1*03:01–DQB1*03:02	1.43	1.82
DQA1*03:01–DQB1*03:05	1.43	1.50
DQA1*03:03–DQB1*02:02	1.32	4.11
DQA1*05:01–DQB1*02:01	14.82	2.00
DQA1*05:05–DQB1*03:01	13.10	2.77

DR7 (DQA1*02:01–DQB1*03:03) and DQA1*05:05–DQB1*03:01 haplotypes, where both genes have higher expression in B lymphocytes than monocytes, and the difference is more pronounced for DQA1 gene (up to seven times more for DQA1*05:01–DQB1*02:01). The third group includes DQB1*06 and DQB1*05:02 haplotypes, where DQA1 gene has higher expression in B lymphocytes, but DQB1 gene has higher expression in monocytes (exception, it is equal for DR15-DQA1*01:02–DQB1*06:02). Summarizing again, there is the lowest expression of DQB1*06 and DQB1*05:02 alleles on the antigen-presenting cells, B lymphocytes (Table 3).

4 | DISCUSSION

4.1 | Gene expression of individual DQ alleles was related to two endogenic controls for correlation with both genetically defined expression (DRA) and activation of immune system (PPIA)

Congruent with their role of being the antigen-presenting cells, we observed almost three times higher expression of DRA gene (a measure of HLA class II-promoting stimuli in a sample) in B lymphocytes compared to whole blood. However, it was surprising to see that DRA mRNA level in monocytes was similar to that in B cells and even slightly higher (the difference was not statistically significant). The reason for this observation remains unknown.

As DRA, including its promoter area, is nonpolymorphic gene, we expected its level to be directly related to the HLA class II-promoting stimuli in each sample. High linear correlation between C_t values of DRA and PPIA gene assays observed in B lymphocytes and monocytes indicated that individuals within our study population were more or less homogenous regarding HLA class II expression in these cell types. To our surprise, we observed good correlation between DRA and PPIA also in samples from whole blood. The donors were healthy individuals, not undergoing acute infection or autoimmune disease. It is thus reasonable to assume that the absence of inflammatory cytokines in their blood and amount of blood cell populations within normal, reference range (not tested) could lead to observed homogeneity. The formula describing the linear relationship was slightly different in each cell type, suggesting differential regulation of HLA class II expression in these cell types.

High correlation between DRA and PPIA transcript levels also makes it evident that using PPIA as an endogenous control for analysis of class II gene and allele expression in healthy people will not lead to huge skewing of data. However, the DRA may come more useful than classical endogenous control when studying HLA expression under conditions of immune stimulation, and it could be interesting to compare DRA and PPIA expression under these conditions.

4.2 | Interallelic and intercell type differences in mRNA expression level of DQA1 and DQB1 gene alleles

We compared differences in expression of DQA1 and DQB1 gene alleles in two cell types, monocytes and B lymphocytes. To obtain a reference frame and compare data with our previous studies, the analysis in whole-blood cells was also included.

According to our results, the relationship between DRA-normalized mRNA expression levels of DQA1 alleles in whole blood is DQA1*03:01, *03:03 > *01:03 > *01:02 (DR13, DR15-linked) > *01:01 > *02:01 > *05:01, *05:05. This hierarchy is just descriptive, as in most of the alleles we did not have enough samples to provide statistically significant results. Hierarchy confirmed by statistics was *01:03, *03:01 > *01:01 > *05:05, which is in accordance with our previous study on DQA1 gene allele expression in whole blood (Zajacova et al., 2015) and with other studies in peripheral blood lymphocytes (Donner et al., 2002; Maffei et al., 1997) or mononuclear cells (PBMC; Britten et al., 2009). The exception to this trend was reported by Fernandez et al. (2003), who observed the highest expression of DQA1*04, and no interallelic differences between other DQA1 alleles in PBMC, and by Pisapia et al. (2016), who reported higher

expression of DQA1*05 allele compared to *01 in monocyte-derived DCs. Essentially, the same expression hierarchy as for whole blood was observed in monocytes (Table 2, Figure 2a).

However, the situation was different in B lymphocytes: although DQA1*03:01 was still the most expressed one, it was surprising to see *05 group alleles among the most expressed alleles as well, as they were the least expressed ones in monocytes in our study and were also consistently reported to be the lowest expressed allele group in various whole-blood cell subsets by others (Britten et al., 2009)(Donner et al., 2002)(Maffei et al., 1997). The higher expression of DQA1*05:01 compared to 01:01 allele was observed only in recent study by Pisapia et al. (2016), where the authors reported DQA1*05 allele mRNA levels 1.9–4.6 higher compared to the transcription of *01:01 allele in heterozygous DQA1*01:01/*05:01 B lymphocyte-derived lymphoblastoid cell lines from both coeliac disease patients and the healthy controls. Thus, the reason for the discrepancy in DQA1*05 allele expression is most probably due to difference in cell types being studied. The same team observed identical DQA1*01:01 > 05:01 relationship in monocyte-derived DCs (Pisapia et al., 2016), which is inconsistent with low expression of DQA1*05 alleles observed in this study; however, the possibility that during the maturation of monocyte-derived DC, different alleles are induced to different levels (Cesari, Caillens, Cadet, Pabion et al., 1999) and expression ratio changes cannot be excluded.

When different cell populations were compared, the DQA1 allele expression was higher in B lymphocytes than in monocytes for each allele. It was also observed that the higher the expression of allele in monocytes, the smaller the difference in its expression between cell types. For example, the level of the most expressed DQA1*03 group alleles was just 1.4 times higher in B cells compared to monocytes; however, the amount of DQA1*05 alleles was around 14 times higher in B cells. Therefore, the expression of alleles in B lymphocytes was more balanced (maximum ratio between mean allele expression of 1.6), while in monocytes, the mean levels of the transcripts of most expressed allele DQA1*03:03 were 10 times higher than those of the least expressed alleles of *05 group.

Expression of 10 DQB1 alleles belonging to DQB1*02, *03, *05 and *06 groups was analysed. Within all three cell types examined, DQB1*02 alleles were the least expressed ones and DQB1*06 alleles the most expressed (Table 2, Figure 2b). The lowest expression of DQB1*02 and highest of *06 allele group were observed also by Britten et al. in PBMC (Britten et al., 2009), and different strength of these promoters was confirmed by expression in transfected B cell lines. Similar to our samples, DQB1*06:02 > 05:01 (5 times of difference) and 05:01 > 03:01 (2 times of difference) hierarchy was observed also in monocytes (Cesari, Caillens, Cadet, Pabion et al., 1999). Beaty, West, & Nepom (1995) observed in B cell lines around 2.5 higher transcription of the heterologous reporter gene under the control of DQB1*03:01 promoter compared to *03:02 promoter. However, in our samples, we observed DQB1*03:02 allele expression slightly higher than that of DQB1*03:01 allele (and the result reached significance in whole-blood cells). Finding of 2.6–9 times higher mean expression of DQB1*02:01 allele compared to the expression of DQB1*03, *05 and *06 mRNA in monocyte-derived DCs and B-LCL (Pisapia et al., 2016) is discrepant with our data, which reveals that DQB1*02:01 allele was the least expressed one in all cell types examined. Other studies examining unbalanced expression of DQB1 alleles used heterozygotes for DQB1*04 allele (Cesari, Caillens, & Cadet, 1999; Nepom, Chung, & West, 1995), which were not present in our study sample. Hence, a comparison of our result with theirs was not feasible.

Relationship between the expressions of DQB1 alleles in different cell types was not so clear as was the case with DQA1 gene. For alleles DQB1*02 group, *03:01, *03:02 and *05:01, the allele expression was higher in B lymphocytes, for DQB1*03:03 and 06:02, allele expression was at the same level, and finally, it was lower in B cells compared to monocytes in DQB1*06:03 and *06:04 alleles. Similar as for DQA1 allele, interallelic expression was much more uniform in B cells (most/least expressed allele means ratio of 3.5) compared to monocytes (ratio of 15.4).

4.3 | Is CIITA responsible for cell type-specific regulation of HLA class II genes?

Polymorphisms were described in promoters of DQA1 and DQB1 genes, affecting either sequence or spacing of conserved X and Y boxes (Beaty et al., 1995; Indovina et al., 1998). These polymorphisms explain some of the observed interallelic differences in the expression of these genes. However, results of our study indicate that promoter polymorphism is not the only mechanism affecting hierarchy in class II allele expression. DQA1*05 alleles were the least expressed alleles in monocytes, although they are among most expressed in B cells (mRNA expression normalized to DRA). Also, amount of DRA gene (normalized to PPIA) was almost the same in both monocytes and B lymphocytes. Therefore, some cell type-specific factor responsible for cell type-specific differences in expression of both HLA class II alleles and isotypes must exist. There are two possible mechanisms that can be responsible for this phenomenon. Some factor needed for transcription could possibly be more abundant in B cells than in monocytes, leading to saturation of all class II promoters and more balanced expression in B cells, while in monocytes lower amount of the factor would lead to competition between promoters. If this was true, then in heterozygotes, the level of relative expression of alleles with "weak" promoter would depend on the promoter strength of the second allele in genotype. However, this is in disagreement with our observations that mRNA level of an DQA1 allele tends to stay constant, irrespective of the identity of the second allele (Zajacova et al., 2015; also confirmed by this study). Second explanation considers different regulatory proteins pres- ent in B cells, but not in monocytes. This function could be exerted by

CIITA itself**.** CIITA is transcribed from four different promoters, resulting in three isoforms of protein that differ by their N-terminal parts (Muhlethaler-Mottet, Otten, Steimle, & Mach, 1997). Each isoform functions in different cell types—activation of promoter I leads to production of CIITA isoform I that regulates constitutive HLA class II expression in dendritic cells and cells of macrophage lineage (with or without IFN-γ induction), promoter III and CIITA isoform III regulate

constitutive expression in B lymphocytes, and isoform IV transcribed from promoter IV is responsible for IFN-γ -inducible CIITA expression in cells of nonhaematopoietic origin (LeibundGut-Landmann et al., 2004; Muhlethaler-Mottet et al., 1997). CIITA isoforms differ not only in their expression pattern, but also in their function. N-terminal acidic domain of CIITA functions as transcription activation domain, and a large number of proteins shown to interact with CIITA employ the residues in or near this domain for binding (Harton & Ting, 2000). Beaulieu, Leon Machado, Ethier, Gaudreau, & Steimle (2016) documented that N-terminal domain of CIITA isoform III is responsible for faster turnover (i.e. lower levels) and higher transactivation potential of isoform III compared to other protein isoforms. Increased activation potential was mostly due to more efficient interaction with transcription machinery and promoter-binding protein (Beaulieu et al., 2016). Therefore, it is possible that promoter polymorphisms, cell type-specific CIITA isoforms and maybe some other, yet unidentified, cell type-specific factors interact and generate observed complexity of HLA class II allele expression.

The rate of transcription is the major determinant of the level of MHC molecule synthesis and expression on the cell surface (Abbas, Lichtman, & Pillai, 2012). Cytokines enhance MHC expression by stimulating the transcription of class I and class II genes in a wide variety of cell types. These effects are mediated by the binding of cytokine-activated transcription factors to DNA sequences in the promoter regions of MHC genes. Several transcription factors are assembled and bind CIITA, and the entire complex binds to the class II promoter and promotes efficient transcription. By keeping the complex of transcription factors together, CIITA functions as a master regulator of class II gene expression.

In conclusion, we described differences in expression of DQA1 and DQB1 gene alleles in whole-blood cells, monocytes and B lymphocytes. Our findings support the idea that expression level of HLA class II alleles is the result of both promoter- and cell-specific factors.

ACKNOWLEDGEMENTS

We thank Katarina Kolostova for useful and inspirational inputs regarding the methodology and the research idea.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS

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 on 25 June 2015 (approval code: CERNA 20150000).

ORCID

M. Cerna http://orcid.org/0000-0002-1208-5588

REFERENCES

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2012). *Cellular and molecular immunology* (7th ed.). ?????: Elsevier Saunders.

Andersen, L. C., Beaty, J. S., Nettles, J. W., Seyfried, C. E., Nepom, G. T., & Nepoom, B. S. (1991). Allelic polymorphism in transcriptional regulatory regions of HLA-DQB genes. *The Journal of Experimental Medicine*, *173*(1), 181–192. https://doi.org/10.1084/jem.173.1.181

Beaty, J. S., West, K. A., & Nepom, G. T. (1995). Functional effects of a natural polymorphism in the transcriptional regulatory sequence of HLA-DQB1. *Molecular and Cellular Biology*, *15*(9), 4771–4782. https://doi.org/10.1128/MCB.15.9.4771

Beaulieu, Y. B., Leon Machado, J. A., Ethier, S., Gaudreau, L., & Steimle, V. (2016). Degradation, promoter recruitment and transactivation mediated by the extreme N-terminus of MHC class II transactivator CIITA isoform III. *PLoS One*, *11*(2), e0148753. https://doi.org/10.1371/journal.pone.0148753

Britten, A. C., Mijovic, C. H., Barnett, A. H., & Kelly, M. A. (2009). Differential expression of HLA-DQ alleles in peripheral blood mononuclear cells: Alleles associated with susceptibility to and protection from autoimmune type 1 diabetes. *International Journal of Immunogenetics*, *36*(1), 47–57. https://doi.org/10.1111/j.1744-313X.2008.00823.x

Cazalis, M., Friggeri, A., Cavé, L., Demaret, J., Barbalat, V., Cerrato, E., & Venet, F. (2013). Decreased HLA-DR antigen-associated invariant chain (CD74) mRNA expression predicts mortality after septic shock. *Critical Care*, *17*(6), R287. https://doi.org/10.1186/cc13150

Cerna, M. (2008). Genetics of autoimmune diabetes mellitus. *Wiener Medizinische Wochenschrift*, *158*(1–2), 2–12. https://doi.org/10.1007/s10354-007-0448-0

Cesari, M., Caillens, H., & Cadet, F. (1999). In vivo analysis of HLA-DQ gene expression in heterozygous cell lines. *Immunogenetics*, *50*, 309–318. https://doi.org/10.1007/s002510050607

Cesari, M., Caillens, H., Cadet, F., Pabion, M., Cesari, M., Cadet, F., & Pabion, M. (1999). In vivo analysis of HLA-DQ gene expression in heterozygous cell lines. *Immunogenetics*, *50*, 309–318. https://doi.org/10.1007/s002510050607

Del Pozzo, G., Perfetto, C., Ombra, M. N., Ding, G. Z., Guardiola, J., & Maffei, A. (1992). DNA polymorphisms in the 5′-flanking region of the HLA-DQA1 gene. *Immunogenetics*, *35*(3), 176–182. https://doi.org/10.1007/BF00185111

Donner, H., Seidl, C., Rau, H., Herwig, J., Seifried, E., Usadel, K. H., & Badenhoop, K. (2002). Unbalanced amounts of HLA-DQA1 allele mRNA: DQA1*03 shows high and DQA1*0501 low amounts of mRNA in heterozygous individuals. *European Journal of Immunogenetics*, *29*(4), 321–330. https://doi.org/10.1046/j.1365-2370.2002.00321.x

Douhan, J., Lieberson, R., Knoll, J. H., Zhou, H., & Glimcher, L. H. (1997). An isotype-specific activator of major histocompatibility complex (MHC) class II genes that is independent of class II transactivator. *The Journal of Experimental Medicine*, *185*(11), 1885–1895. https://doi.org/10.1084/jem.185.11.1885

Fernandez, S., Wassmuth, R., Knerr, I., Frank, C., & Haas, J. P. (2003). Relative quantification of HLA-DRA1 and -DQA1 expression by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). *European Journal of Immunogenetics*, *30*(2), 141–148. https://doi.org/10.1046/j.1365-2370.2003.00376.x

Harton, J. A., & Ting, J. P. (2000). Class II transactivator: Mastering the art of major histocompatibility complex expression. *Molecular and Cellular Biology*, *20*(17), 6185–6194. https://doi.org/10.1128/MCB.20.17.6185-6194.2000

Indovina, P., Megiorni, F., Ferrante, P., Apollonio, I., Petronzelli, F., & Mazzilli, M. C. (1998). Different binding of NF-Y transcriptional factor to DQA1 promoter variants. *Human Immunology*, *59*(12), 758–767. https://doi.org/10.1016/S0198-8859(98)00082-2

Kobr, M., Reith, W., Herrero-Sanchez, C., & Mach, B. (1990). Two DNA-binding proteins discriminate between the promoters of different

members of the major histocompatibility complex class II multigene family. *Molecular and Cellular Biology*, *10*(3), 965–971. https://doi.org/10.1128/MCB.10.3.965

Laupeze, D., Fardel, O., Onno, M., Bertho, N., Drenou, B., Fauchet, R., & Amiot, L. (1999). Differential expression of major histocompatibility complex class ia, ib and ii molecules on monocytes and monocyte-derived dendritic and macrophagic cells. *Human Immunology*, *60*, 591–597. https://doi.org/10.1016/S0198-8859(99)00025-7

LeibundGut-Landmann, S., Waldburger, J.-M., Krawczyk, M., Otten, L. A., Suter, T., Fontana, A., & Reith, W. (2004). Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *European Journal of Immunology*, *34*(6), 1513–1525. https://doi.org/10.1002/(ISSN)1521-4141

Lenormand, C., Bausinger, H., Gross, F., Signorino-Gelo, F., Koch, S., Peressin, M., … Tourne, S. (2012). HLA-DQA2 and HLA-DQB2 genes are specifically expressed in human Langerhans cells and encode a new HLA class II molecule. *Journal of Immunology*, *188*(8), 3903–3911. https://doi.org/10.4049/jimmunol.1103048

Maffei, A., Harris, P. E., Reed, E. F., Del Pozzo, G., Ciullo, M., Suciu-Foca, N., & Guardioloa, J. (1997). Differential expression of insulin-dependent diabetes mellitus-associated HLA-DQA1 alleles in vivo. *European Journal of Immunology*, *27*, 1549–1556. https://doi.org/10.1002/(ISSN)1521-4141

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, *16*(3), 1215. https://doi.org/10.1093/nar/16.3.1215

Morzycka-Wroblewska, E., Harwood, J. I., Smith, J. R., & Kagnoff, M. F. (1993). Structure and evolution of the promoter regions of the DQA genes. *Immunogenetics*, *37*(5), 364–372. https://doi.org/10.1007/BF00216801

Morzycka-Wroblewska, E., Munshi, A., Ostermayer, M., Harwood, J. I., & Kagnoff, M. F. (1997). Differential expression of HLA-DQA1 alleles associated with promoter polymorphism. *Immunogenetics*, *45*(3), 163–170. https://doi.org/10.1007/s002510050185

Muhlethaler-Mottet, A., Otten, L. A., Steimle, V., & Mach, B. (1997). Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *The EMBO Journal*, *16*(10), 2851–2860. https://doi.org/10.1093/emboj/16.10.2851

Nepom, G. T., Chung, J., & West, K. A. (1995). Differential expression of HLA-DQB1 alleles in a heterozygous cell line. *Immunogenetics*, *42*(2), 143–148.

Otten, L. A., & Steimle, V. (1998). Quantitative control of MHC class II expression by the transactivator CIITA. *European Journal of Immunology*, *28*, 473–478. https://doi.org/10.1002/(ISSN)1521-4141

Perfetto, C., Zacheis, M., McDaid, D., Meador, J. W., & Schwartz, B. D. (1993). Polymorphism in the promoter region of HLA-DRB genes. *Human Immunology*, *36*(1), 27–33. https://doi.org/10.1016/0198-8859(93)90005-L

Pisapia, L., Camarca, A., Picascia, S., Bassi, V., Barba, P., Del Pozzo, G., & Gianfrani, C. (2016). HLA-DQ2.5 genes associated with celiac disease risk are preferentially expressed with respect to non-predisposing HLA genes: Implication for anti-gluten T cell response. *Journal of Autoimmunity*, *70*, 63–72. https://doi.org/10.1016/j.jaut.2016.03.016

Reith, W., LeibundGut-Landmann, S., & Waldburger, J.-M. (2005). Regulation of MHC class II gene expression by the class II transactivator. *Nature Reviews Immunology*, *5*(10), 793–806. https://doi.org/10.1038/nri1708

Ting, J. P., Trowsdale, J., Hill, C., Carolina, N., & Allcock, F. (2002). Genetic control of MHC class II expression. *Cell*, *109*, S21–S33. https://doi.org/10.1016/S0092-8674(02)00696-7

Vincent, R., Louis, P., Gongora, C., Papa, I., Clot, J., & Eliaou, J. F. (1996). Quantitative analysis of the expression of the HLA-DRB genes at the transcriptional level by competitive polymerase chain reaction. *Journal of Immunology*, *156*(2), 603–610.

Yu, L., & Sheehy, M. (1991). The cryptic HLA-DQA2 ("DX alpha") gene is expressed in human B cell lines. *Journal of Immunology*, *15*(147), 4393–4397.

Zajacova, M., Kotrbova-Kozak, A., Cepek, P., & Cerna, M. (2015). Differences in promoter DNA methylation and mRNA expression of individual alleles of the HLA class II DQA1 gene. *Immunology Letters*, *167*(2), 147–154. https://doi.org/10.1016/j.imlet.2015.08.006

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Zajacova M, Kotrbova-Kozak A, Cerna M. Expression of HLA-DQA1 and HLA-DQB1 genes in B lymphocytes, monocytes and whole blood. *Int J Immunogenet*. 2018;00:1–10. https://doi.org/10.1111/iji.12367