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Bc. Marek Černoch

Studium epigenetické regulace HLA genů II. třídy na úrovni histonových modifikací

The study of epigenetic regulation of HLA class II genes at the level of histone modifications

Diplomová práce

Školitel: doc. MUDr. Marie Černá, CSc.

Praha, 2014

Declaration

I declare that I wrote this work myself and that I stated all information sources and literature I used. This work or its significant part was not submitted in order to obtain another or the same academic title.

In Prague, 8. 8. 2014

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 8. 8. 2014

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Bc. Marek Černoch

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Abstract

Introduction: The epigenetic modifications can significantly affect and alter the gene activity by regulating their expression, having direct impact on various processes in human body. Epigenetic processes are involved in ethiopathogenesis of many diseases. From this point of view, MHC genes are very important as they were linked to many autoimmune disorders, for example type 1 diabetes mellitus. In general autoimmune diseases appear to be connected to certain MHC class II genes.

Aims: The aim of this thesis is to determine the relationship between expression levels and histone modifications present in the promoter area of MHC class II gene, DQA1. Moreover, we also analyze and compare the DQA1 gene mRNA expression depending on the QAP promoter allele.

Methods: We isolated both nucleic acids (DNA and RNA) and leukocytes from peripheral blood samples collected from voluntary donors. DNA was utilized for genotypization of individuals. RNA was subjected to reverse transcription and the quantitative PCR was performed in order to determine the level of expression. Leukocytes were used for chromatin immunoprecipitation, which was evaluated using quantitative PCR.

Results: The expression level of QAP allele 3.1 was found to be higher than for the rest of the alleles Allele 4.1A showed, on the other hand, expression significantly lower. Histone modifications were measured for DQA1 alleles *01, *02, *03 and *05. No statistically significant relationship between allelic expression and histone modifications present was found.

Keywords: MHC class II, DQA1, epigenetics, histone modification, RNA expression, genotypization

Abstrakt

Úvod: Epigenetické modifikace mohou mít významný vliv na na aktivitu genů regulací jejich exprese, čímž mohou přímo ovlivnit mnohé procesy v lidském těle. Epigenetické procesy se také podílejí na etiopatogenezi mnoha chorob. Z tohoto úhlu pohledu se MHC geny jeví jako zvláště důležité, protože jsou spojeny s mnoha autoimunitními poruchami, například s diabetes mellitus prvního typu. Autoimunitní choroby se obecně zdají být úzce propojeny s určitými MHC geny II. třídy.

Cíle: Cílem této práce je určit vztah mezi úrovní exprese a přítomností histonových modifikací v promotorovém úseku MHC genu II. třídy DQA1. Dále analyzujeme a srovnáváme exprese mRNA genu DQA1 v závislosti na přítomné QAP alele promotoru.

Metody: Ze vzorků periferní krve od dobrovolných dárců jsme izolovali nukleové kyseliny (DNA i RNA) a leukocyty. DNA byla nadále využita pro genotypizaci jedinců. RNA byla podrobena reverzní transkripci a následně kvantitativní PCR za účelem vyhodnocení úrovně exprese. Leukocyty byly použity pro chromatinovou imunoprecipitaci, která byla vyhodnocena pomocí kvantitativní PCR.

Výsledky: Úroveň exprese QAP alely 3.1 byla zvýšená oproti ostatním alelám. Alela 4.1A se naopak exprimovala na významně nižší úrovni. Histonové modifikace byly zjištěny pro DQA1 alely *01, *02, *03 a *05. Mezi expresí a přítomnými histonovými modifikacemi však nebyl nalezen žádný statisticky významný vztah

Klíčová slova: MHC II. třídy, epigenetika, histonové modifikace, RNA exprese, genotypizace

Contents

List of abbreviations

APC	antigen-presenting cells
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
EDTA	ethylenediaminetetraacetic acid
HLA	human leukocyte antigen
HSP70	heat-shock protein 70
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
piRNA	Piwi-interacting ribonucleic acid
RCLB	red cell lysis buffer
SDS	sodium dodecyl sulfate
TNF-α	tumor necrosis factor -α
WCLB	white cell lysis buffer

1. Introduction

The epigenetics, as the way to alter the activity and functionability of genes without changing the actual genetic code and with the possibility to pass these alterations to the progeny, can be counted amongst newer fields of interest, as it was named and defined for the first time only several decades ago. Soon it became clear that epigenetic mechanisms, including for example DNA methylation or histone modification, can have a significant impact on our lives.

It is known for some time now that autoimmune diseases are often influenced by the environment we live in as much as by our genetic predisposition, it was however not clear how can these factors interact in autoimmune disease outbreak, or how can one prevent the other from starting it on its own. This mystery has only begun to unravel in recent years, pointing at epigenetic mechanisms as possible answer. These mechanisms were shown to be influenced by external factors while having significant influence on basic biological processes.

From genes known to affect autoimmune disorders, MHC genes are especially significant. Being the key to specific antigen recognition and thus being crucial for adaptive immunity mechanisms, any changes can have a great influence on our capability to deal with potential threats, both external and internal. Our research group is studying MHC class II genes DRB1, DQA1 and DQB1, their polymorphisms, epigenetic regulation and possible connection to type 1 diabetes mellitus.

The purpose of this thesis is to study the activity of allelic variants of the DQA1 gene in healthy subjects. For this purpose, blood samples were collected from volunteers and further processed. That includes the genotypization from isolated DNA to identify DRB1, DQA1 and DQB1 alleles carried. In order to determine their relative activity two main analyses have been performed. First, gene expression of DQA1 alleles was determined at the level of mRNA quantification. Second, isolated leukocytes were subjected to the chromatin immunoprecipitation to study the histone modifications in promoter DQA1 region. Last but not least, comparison of obtained data with the results of mRNA expression level measurements was investigated.

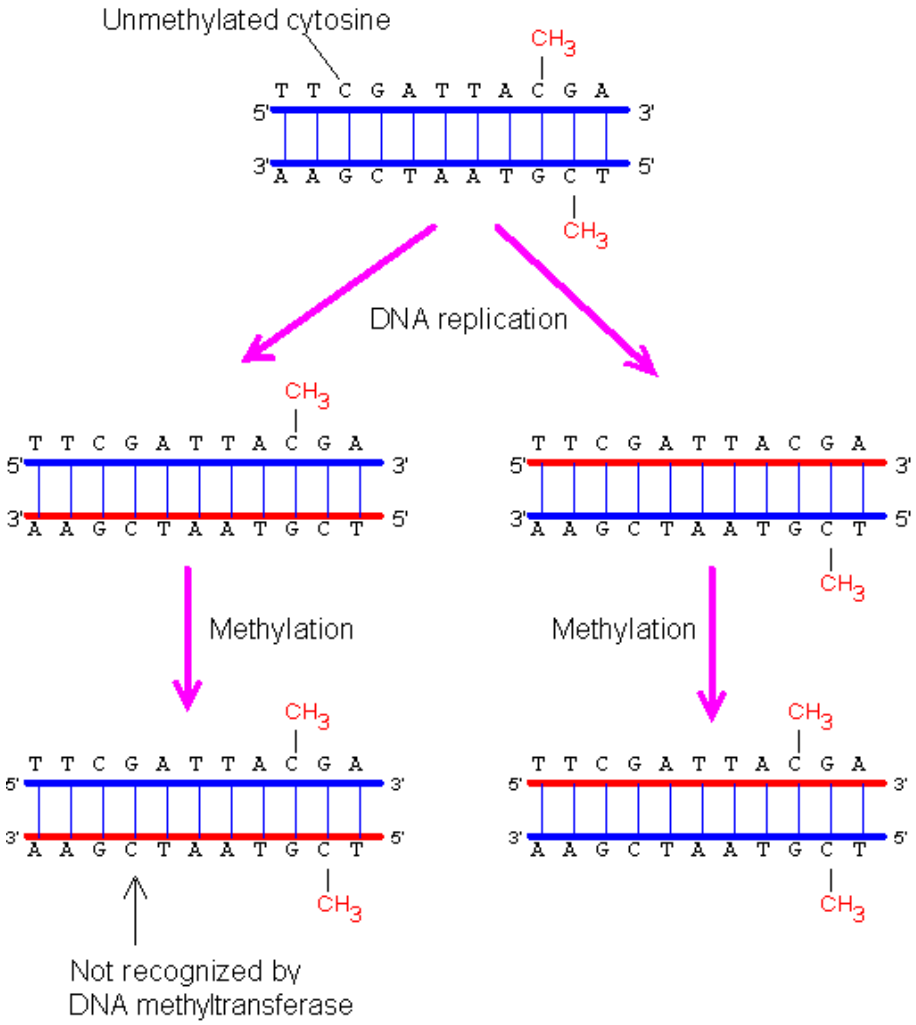
2. Literature review

2.1. Epigenetics

The term „epigenetics“ describes the study of changes in phenotype or gene expression that are not based on DNA sequence and can be heritable. The term itself was first used by C. H. Waddington (2012), geneticist and embryologist who performed significant studies on the development of Drosophila wings. Epigenetic regulation of the genome is important for maintaining its stability and proper development and differentiation of the cell. This regulation can be influenced by external factors, as well as aging processes. Various cellular mechanisms are currently included in the category of epigenetic processes, including crucial events connected to organism development, such as gene imprinting or X chromosome inactivation (Payer, Lee, & Namekawa, 2011).

2.1.1. DNA methylation

It is important to notice that while DNA methylation is fundamental for vertebrates, significantly lower amounts of methylated DNA were found in insect (Bird et al., 1995). Many bacteria, on the other hand, possess unique DNA adenine methylase, capable of adding methyl group on adenine in specific GATC sequence (van Steensel & Henikoff, 2000). It is, therefore, clear that described epigenetic mechanisms are not universally applicable. The most basic mechanism of epigenetic regulation, DNA methylation is accomplished by transferring the methyl group from S-adenosyl methionin to cytosine, leading to the creation of 5-methylcytosine. This process is specific for CpG sequence. The percentage of methylated CpGs in DNA strand varies depending on the type of tissue (Ehrlich et al., 1982). Methylation itself is performed by proteins called DNA methyltransferases (Dnmt). Some of them – Dnmt3a and Dnmt3b - are active during embryogenesis and cell differentiation, performing *de novo* methylation which is crucial for embryonic development. They have, however, no impact on the maintenance of already methylated patterns (Okano, Bell, Haber, & Li, 1999). That is dependent on another type of DNA methylases, namely Dnmt1, with its activity being connected to DNA replication. (Pic.1) Once there, Dnmt1 is able to recognize methylation of CpG sites on parent strand and subsequently catalyses the methylation of corresponding CpG sites on newly replicated strand (Leonhardt, Page, Weier, & Bestor, 1992).



Pic.1 - The mechanisms on the DNA methylation heritability. DNA methyltransferase 1 (not shown) only adds methyl group to cytosine in CG pair, where corresponding CG pair on opposing strand is already methylated (http://www.web-books.com/MoBio/Free/Ch7F2.htm)

In 5‘ region of many genes, often in promoters, there are specific groups of unmethylated CpGs, called „CpG islands“. They are defined as a regions longer than 500 base pairs with more than 55% CG content, also CpG to GpC ration must be higher than 0,6 (Takai & Jones, 2003). The methylation of these islands does not always correlate with the activity of the given promoter and are often unmethylated in genes that show increased levels of lysine 4 dimethylation of histone H3, suggesting a relationship between CpG island methylation and this specific chromatin posttranscriptional modification (Weber et al., 2007).

DNA methylation is an important mechanism for the regulation of the transcription, having significant influence on the activity of the gene expression. Certain transcription factors are unable to bind to DNA strand in case it is methylated (Comb & Goodman, 1990). Another

type of transcription regulation is performed by methylation dependent binding proteins – MDBPs – which are possibly linked to gene silencing (both specific and chromosome-wide) and heterochromatin formation. Unlike the aforementioned example, these factors interact primarily with methylated DNA. They have a typical methyl-CpG binding domain, allowing them to interact specifically with the methylated DNA strand (Nan, Meehan, & Bird, 1993), and a transcriptional repression domain. They appear to be of primary significance for the transcription regulation, compared to direct suppression of transcription factor binding by methylation (Nan, Campoy, & Bird, 1997).

2.1.2. The chromatin structure and post-translational modifications of histones

Chromatin is a structure common to all eukaryotes that provides a scaffold for basic processes like DNA replication and transcription. Two forms of chromatin are generally described. Firstly, heterochromatin, condensed form which can be found in abundance during mitosis and meiosis and mostly lacks DNA regulating activity. Secondly, euchromatin, decondensed form providing suitable environment for DNA regulation. There are notable differences in euchromatin/heterochromatin ratio between differentiated somatic cells and pluripotent stem cells, with more chromatin in decondensed form present in stem cells. This correlates with significantly higher levels of transcription, both in coding and non-coding regions (Gaspar-Maia, Alajem, Meshorer, & Ramalho-Santos, 2011). The basic unit of chromatin is a nucleosome. Each nucleosome represents 147 base pairs long sequence of DNA strand, wrapped in two turns around the complex of eight core histone proteins – dimers of H2A, H2B, H3 and H4. There are also H1 histones present, known also as „linkers“. Their function is to bind at entry and exit sites and lock the DNA on the octamer, allowing the creation of higher order structures (Eickbush & Moudrianakis, 1978). Each of the core histones contains N-terminal „tail“, flexible structure extending outside the histone-DNA complex, which is a significant target area for post-transcriptional modifications and due to its binding capabilities it can also potentially modify higher order structure of the nucleosome. The post-transcriptional modifications and remodeling of chromatin structure itself can appear simultaneously, cooperating towards common goal. One notable example of such a situation would be DNA double-strand break repair process (Gospodinov & Herceg, 2013).

Post-translational modifications are one of the most important epigenetic mechanisms and play an important part in activating or silencing specific genes, affecting many vital processes, for example the cellular differentiation (Clark & Felsenfeld, 1971) or genome stability maintenance (Peters et al., 2001). Most significant post-transcriptional modifications found mostly on histone tails include acetylation, methylation, phosphorylation and ubiquitination; there are several more that appear less frequently, these include sumoylation, citrullination and ADP-ribosylation. These modifications are thought to appear in certain combinations, creating the so-called „histone code“ (Strahl & Allis, 2000).

There is no general rule which could be applied universally to decode the histone code; certain modifications on certain amino acids are however connected with certain levels of transcription. It was thought initially that methylation of lysine residues causes repression of transcription, it was later determined though that in reality it is not that simple. Studies have shown that it also depends not only on the position of given lysine, but also on the number of methyl groups present. Good example is lysine 4 on histone H3. Lysine methylation in this particular case can result in three states: mono-, di- and trimethylation. While dimethylated lysine 4 was found in both transcriptionally active and inactive euchromatic regions, trimethyl residue was present exclusively in active sites. Therefore, trimethylated lysine 4 appears to be connected to the active state of gene expression contrary to general rule (Santos-Rosa et al., 2002). It was also shown that different types of methylation can be connected to different sites of chromatin (Rice et al., 2003). Methylation itself is performed by a family of proteins called histone methyltransferases. It is worth noting that histone methyltransferases can be further divided to two groups depending on whether they transfer methyl groups to lysine or arginine, as arginine is the second amino acid capable of being methylated (Clarke, 2013).

Histone acetylation is another type of post-translational modification which plays a crucial role in regulating gene expression and is, similarly to methylation, catalyzed by specific proteins, histone acetyltransferases. Similarly, histone deacetylases are proteins capable of removing acetyl groups from histones. Acetylation can occur at lysines on N-terminal histone tails, which leads to neutralization of its otherwise positive polarity of histone proteins and the increase of hydrophobicity (Kuo & Allis, 1998). That results in the weakening of its bond

to negatively charged DNA strand and leads to increased accessibility for transcription factors. However, unlike methylation, histone acetylation plays yet another important role, as studies suggest it participates directly in nucleosome assembly during replication. During that process, H3/H4 tetramer binds to the DNA, followed by the formation of H2A/H2B dimers. Histone subunit H4 is commonly acetylated on its N-terminal domain and frequently interacts with other subunits, mainly H2A. Acetylation can influence these interactions, having possibly impact on the final structure of assembled chromatin (Lee, Wei, & Lee, 2011).

2.1.3. The relationship between DNA methylation and chromatin structure

There is a distinct relationship between the methylation of CpG islands in promoter regions and chromatin configuration. When the islands are not methylated, usually the chromatin structure supports active transcription as well. That includes significantly heightened levels of acetylation on histones H3 and H4 and reduced amount of histone H1. Also, nucleosome-free regions can be found (Tazi & Bird, 1990).

2.1.4. RNA interference

RNA interference is a process of gene expression inhibition using specific complementary small RNA molecules. Several types of RNA are recognized to have the ability to participate in this process, for example siRNA (small interfering RNA) or miRNA (microRNA). In recent years, a relationship was found between RNA interference and chromatin formation. Double-stranded RNA containing promoter sequence has been shown to be capable of initiating *de novo* methylation of plant promoters and causing transcriptional silencing of the gene (Mette, Aufsatz, van der Winden, Matzke, & Matzke, 2000). Similarly, experiments performed on mice suggested a link between piRNA (Piwi-interacting RNA) and transposon methylation in germ cells (Aravin et al., 2008). RNA interference is clearly tied to heterochromatin formation as well, since several studies have shown its connection to the methylation of lysine 9 on histone H3 (Pal-Bhadra et al., 2004; Volpe et al., 2002).

2.2. Major histocompatibility complex

The major histocompatibility complex (MHC) is a group of highly polymorphic genes, significant for the correct function of immune system. It encodes a large group of cell surface molecules present in all vertebrates. The main function of these molecules is to present peptides to T-lymphocytes and by this way to cause their activation or tolerance status. Several classes of MHC can be distinguished.

MHC class I glycoproteins can be found on all nucleus-containing cells, their function is to present peptides of cytosolic origin (both its own and foreign peptides) on the cell surface. MHC class I - peptide complexes are recognized by CD8 positive („cytotoxic“) T-lymphocytes, which in turn are able to trigger apoptotic pathway. This is a significant process for the effective defense against intracellular pathogens such as viruses. The MHC class I structure consists of α chain containing three extracellular domains, and also transmembrane and intracellular parts, and β2 microglobulin subunit. The chain of β2 microglobulin is encoded by its own special gene.

MHC class II glycoproteins are under normal conditions found only on specialized antigen-presenting cells (APC), including dendritic cells, macrophages and B-lymphocytes. These cells can process the proteins of extracellular origin and present their fragments on the cell surface. Other cell types, such as fibroblasts or vascular endothelial cells, are able to express MHC class II proteins under cytokine stimulation. MHC class II - peptide complexes are recognized by CD4 positive („helper“) T-lymphocytes, which in turn leads to T-lymphocytes activation and B-lymphocytes maturation connected with antibody production. The MHC class II structure consists of α and β chains, each of them being composed of two extracellular domains, plus transmembrane and intracellular parts.

Apart from these transmembrane molecules, MHC class III soluble glycoproteins were also described. This group includes secreted proteins with non-antigen-specific immunological functions, such as several components of the complement system (C2, C4) or certain cytokines, tumor necrosis factors and heat shock proteins (TNF-α, HSP70) (Cameron, Tabarias, Pulendran, Robinson, & Dawkins, 1990). Some authors further differentiate between MHC class III and class IV glycoproteins (Gruen & Weissman, 2001). When studying humans, MHC molecules are designated as the human leukocyte antigens (HLA).

2.2.1. MHC class I structure

The MHC class I molecule is constituted by two polypeptide chains, α and β. Main α chain is polymorphic and is comprised of three domains, α1, α2 and α3. The gene for α chain is encoded by MHC region, whereas the gene for β chain – β2 microglobulin – is located separately, on chromosome 15. Both chains are connected via noncovalent interaction of β2 microglobulin and the α3 domain, which possesses relatively conserved structure compared to other two domains (Lopez de Castro, Barbosa, Krangel, Biro, & Strominger, 1985). The groove for presented peptide is located between α1 and α2 domains. Peptides are bound to MHC molecule in endoplasmic reticulum, after being cleaved by proteasome to the length of 8-9 amino acids. The groove has a limited space in MHC class I molecules and, being closed at both ends, cannot therefore bind longer peptides. It is worth noting that only by binding peptide in its groove reaches the MHC class I molecule its full stability. While awaiting the peptide in endoplasmic reticulum, it is stabilized by interactions with chaperone proteins, such as calreticulin or tapasin (dedicated chaperone protein, connecting peptide translocation into endoplasmic reticulum with the directing towards MHC class I molecule) (Neefjes, Jongsma, Paul, & Bakke, 2011).

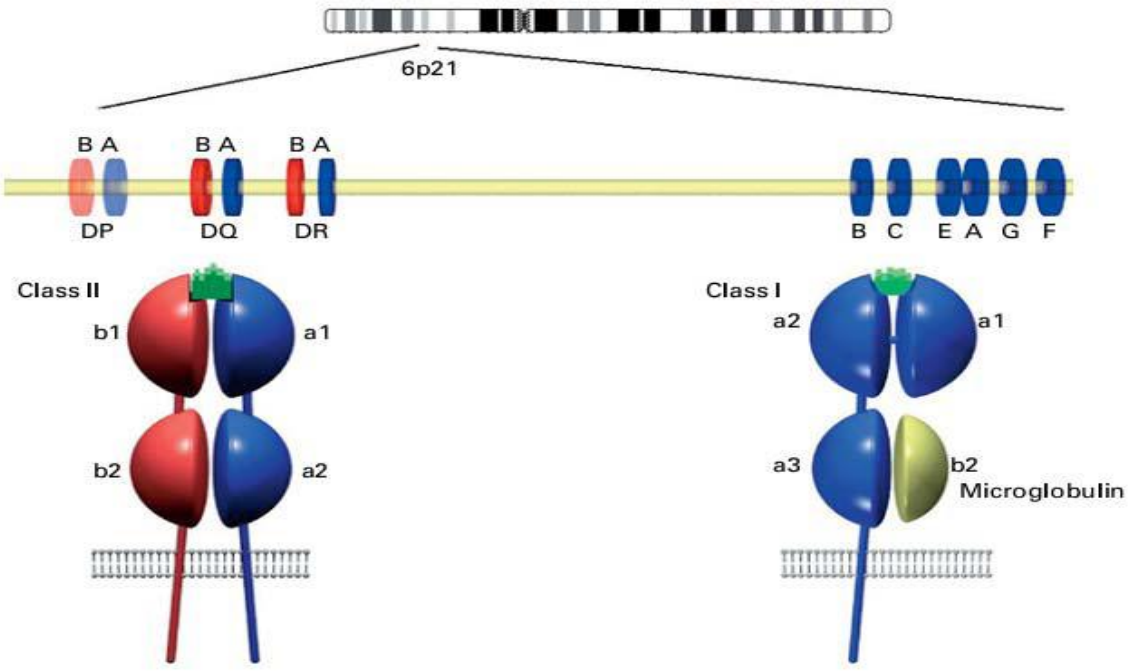
2.2.2. MHC class II structure

The structure on MHC class II molecule is also that of a heterodimer, containing α and β chains that are however, unlike HMC class I chains, both encoded by MHC genes. Each chain is comprised of two domains, α1 and α2 (β1 and β2, respectively). Both chains are, similarly to MHC class I chains, connected via noncovalent interactions. The binding groove is created by α1 and β1 domains and it is not closed on its ends, allowing larger peptide fragment to interact and subsequently be presented. The usual length of presented peptide can therefore vary and usually is up to 20 amino acids (McFarland & Beeson, 2002). During proteosynthesis in endoplasmic reticulum, the peptide-binding groove is blocked by a trimeric polypeptide called invariant chain (also Ii). This stabilizes the molecule and also prevents it from binding any other peptide from its surroundings (that being a role of MHC class I molecules) (Roche & Cresswell, 1990). It also contains a targeting motif, directing MHC class II/Ii complex towards Golgi apparatus and further, to endosomes (Bakke & Dobberstein, 1990). Here, the invariant chain undergoes proteolysis facilitated by cathepsins, only leaving behind a small fragment called class II-associated Ii peptide (CLIP).

This fragment continues to block the binding groove until it is released by HLA-DM protein and replaced by its final ligand (Sloan et al., 1995).

2.2.3. The polymorphism of MHC region

Studies of human genome showed that MHC region, with length of approximately 4 Mbps, is located on chromosome 6. (Pic.2) Typical for this region is high gene density and high polymorphism. Distinct loci can be further distinguished. MHC class I region encodes A, B and C transmembrane glycoprotein single chains; MHC class II region encodes DP, DQ and DR transmembrane glycoprotein double chains and also contains DM and DO loci, whose products help facilitate the binding of peptides to MHC class II in endosomes (Majumder & Boss, 2011). MHC glycoproteins are unique, as they are significantly more polymorphic than any other human protein. This diversity is positively supported by evolutionary selection mechanisms. The advantage of increased rate of heterozygosity is the increased ability of antigen recognition and presentation, allowing the organism to react more effectively to the constant evolution of pathogens (Kim & Polychronakos, 2005).



Pic.2 - MHC region on chromosome 6. Each class II molecule consists of α and β chains, encoded by their respective genes. Class I β chain is created by microglobulin molecule, encoded elsewhere, on chromosome 15. (Kim & Polychronakos, 2005)

MHC polymorphism can be linked to several autoimmune disorders, increasing or lowering the chance of their manifestation (Cruz-Tapias et al., 2012). Examples of MHC-influenced diseases, that were linked to specific MHC haplotypes more than 40 years ago, include myasthenia gravis, typical symptom of which is the dysfunction of acetylcholine receptors on neuromuscular junctions (Pirskanen, Tiilikainen, & Hokkanen, 1972), systemic lupus erythematosus (Dostal, Ivanyi, Macurova, Hana, & Strejcek, 1977) or rheumatoid arthritis (Stastny, 1978), and lately type 1 diabetes (Rotter, 1981)). Usually, affinity to autoimmune diseases is connected to certain alleles of DQ and DR genes of MHC class II region. It is, however, problematic to assign specific contribution and importance to any given gene due to significant effect of linkage disequilibrium – a preferential association of neighboring alleles, correlating with their common descendant from single ancestral chromosome, that are often inherited as a block (Reich et al., 2001). Therefore, entire haplotypes (groups of neighboring alleles) are taken into consideration when trying to map the influence on diseases (Kim & Polychronakos, 2005).

2.2.4. The role of MHC polymorphism in autoimmune disorders

As mentioned above, certain variants of MHC alleles can support the development of several autoimmune disorders. There are more factors playing a role, for example gender (as women has been shown to be more prone to suffer from many autoimmune diseases (Shoenfeld, Tincani, & Gershwin, 2012), but the genetic background is among the most prominent. From this point of view, MHC genes are especially important. MHC class II haplotypes DQ2.5 (alleles DQA1*0501 - DQB1*0201) and DQ8 (alleles DQA1*0301/02 - DQB1*0302) play a significant role as a screening marker when celiac disease and type 1 diabetes are concerned, having a strong predictive value towards the disease manifestation in the individual carrying this genetic heritage (Rotter, 1981)van Beek et al., 2013). The involvement of both class I and class II MHC genes was further discovered, as positive associations were found not only with DR3 (alleles DRB1*0301 – DQA1*0501 – DQB1*0201), but also B8 haplotypes (allele B*0801) (Vieira et al., 1993). The other haplotype like DQB1*0302 – DRB1*04 were also found to be correlated in newer findings (Fekih-Mrissa, Klai, Zaouali, Gritli, & Mrissa, 2013). Studies have, however, shown that the influence of MHC haplotype can vary depending on other factors, such as age or ethnicity (Sylvia, Samuel, Luis, & Zuzet, 2013).

It is, however, not as simple as pointing out specific alleles as "problematic". As many allelic variants can increase the risk of developing an autoimmune disorder, there are other alleles that can lower the risk as well. For example, DR1 haplotype (alleles DRB1*0101 – DQA1*0101 – DQB1*0501) has been shown to correlate with lower occurrence of aforementioned myasthenia gravis (Vieira et al., 1993). Similarly, recent study conducted in Japan discovered that DRB1*1302 allele is associated negatively with rheumatoid arthritis, providing "protective" effect for its carrier (Oka et al., 2014).

2.2.5. The relationship between MHC alleles and type 1 diabetes

Type 1 diabetes, also known as insulin-dependent or juvenile diabetes, is a disorder that is caused by autoimmune destruction of pancreatic β cells, located in the islets of Langerhans, which are responsible for insulin production. The antibodies against β cells circulating in the body are detectable (Cerna et al., 2007). It is also accompanied by T- and B-lymphocytic infiltration (Novota, Cejkova, Cerna, & Andel, 2004). This inevitably leads to the increase of glucose levels in blood and, when not treated properly, can progress to severe complications, such as retinopathy or renal failure. It is one of the most common chronic diseases among children and young adults (Schranz & Lernmark, 1998). The incidence of type 1 diabetes among children seems to have risen significantly during second half of 20th century, generally following linear-increase pattern ever since, varying population from population (Gale, 2002). The susceptibility to this disease is influenced by both environmental and genetic factors. Type 1 diabetes was shown to be a polygenic disorder, influenced both by HLA class I/II genes (Demaine, Hibberd, Mangles, & Millward, 1995) and other loci (Davies et al., 1994). Especially influential seem to be HLA class II genes DQA1, DQB1 and DRB1. Most risky are haplotypes DR3 (DRB1*0301 – DQA1*0501 – DQB1*0201) and DR4 (DRB1*0401/05 – DQA1*0301 – DQB1*0302) (Pugliese & Eisenbarth, 2004).

2.3. The epigenetics of autoimmune disorders

Although the role of epigenetic mechanisms is crucial in the expressivity of genes and the amount of evidence of its importance in disease (especially cancer) is growing, this area of research has been quite neglected until recently, with almost no data being systematically collected at the genome level. The current approach is more focused on DNA sequence variants, disregarding possible influence on disease progressiveness that epigenetic variations can possess (Bjornsson, Fallin, & Feinberg, 2004). Yet, the inactivation of tumor

suppressor genes caused by the hypermethylation of CpG islands in promoter regions has been firmly established and such epigenetic modification has been found in many tumor types - first such discovery was made more than a hundred years ago (Esteller, 2002). There are also other diseases where epigenetic mechanisms play smaller or larger role. Among those we can count for example imprinting disorders as Prader-Willi and Angelman syndromes, or Beckwith-Wiedemann syndrome (Ballestar, Esteller, & Richardson, 2006).

Autoimmune diseases can be also influenced by epigenetic regulation - epigenetic mechanisms are generally considered to be the link between genetic and environmental factors. Autoimmune rheumatic diseases like systemic lupus erythematosus or rheumatic arthritis have been shown to be connected to significant DNA methylation pattern alterations; tissue specificity is also important, as opposed to genetic analysis (Ballestar, 2011). Study performed on monozygotic twins revealed general decrease of 5-methylcytosine present in patients with systemic lupus erythematosus; there were methylation alterations found in CpG-rich regions of the ribosomal DNA, affecting 18S and 28S genes (Javierre et al., 2010). Histone modifications are naturally just as significant. Experiments on models for systemic lupus erythematosus using histone deacetylase inhibitors suggested that several disease-associated genes have their expression affected due to histone deacetylation (Reilly et al., 2004). Similarly, significant increase in histone acetylation has been found in synovial tissue of patients with rheumatoid arthritis, caused by lowered activity of histone deacetylases (Huber et al., 2007).

Studies on monozygotic twins proved to be invaluable in epigenetic research, allowing scientists to compare two individuals with identical genetic background. The epigenetic changes are increasing with age and cause the siblings to be more and more genetically different. This is suspected to play a role in the development of many diseases; therefore there are studies that are trying to use this to uncover more about the factor influencing certain conditions. For example, recent study of psoriasis on monozygotic twins showed differences in gene expression correlated with DNA methylation to be connected with the disease (Gervin et al., 2012).

Monozygotic twins were also used in study researching type 1 diabetes, which examined over a hundred different CpG sites for methylation. Results indeed showed significant

differences in methylation status. Among genes included (and affected) was also HLA-DQB1 (Rakyan et al., 2011). Miao et al. (2012) examined post-translational modifications of histone proteins in genes linked to type 1 diabetes, including HLA-DQB1 and HLA-DRB1, in monocytes and discovered variations, between patients and controls, in acetylation of Lysine 9 on H3 histone along the upstream promoter regions of these two genes. This suggests that the regulation of these genes could be dependent on the acetylation status of histone 3, which in turn could be influenced by type 1 diabetes. In earlier study the same scientist analyzed the status of dimethylation on Lysine 9 on H3 histone in lymphocytes between patients and controls and revealed statistical significance of histone dimethylation in promoter region of CTLA4, another important gene connected to type 1 diabetes (Miao et al., 2008). In conclusion, research results show solid link between type 1 diabetes and epigenetic modifications and it is apparent that epigenetic studies are crucial for full understanding of etiology and pathogenesis of not only this, but also other autoimmune disorders.

3. The aims of the thesis

This master's thesis is a part of a larger ongoing research conducted in the Department of General Biology and Genetics on Third Faculty of Medicine, Charles University. This research focuses on the influence the epigenetic regulation of MHC class II genes has on type 1 diabetes mellitus, working with both healthy subjects and diabetics. The basic hypothesis of this paper is that the epigenetic modifications have a significant impact on the MHC class II genes expression and therefore increase the allelic variability, given by DNA sequence. Main aims pursued in this thesis are:

- Genotypization of MHC class II genes performed on DNA isolated from peripheral blood of healthy volunteers
- The analysis of DQA1 gene mRNA expression and comparison between QAP haplotypes
- The analysis of the relationship between DQA1 expression levels and histone modifications present in the promoter area of DQA1 gene

4. Material

4.1. Chemicals

Distilled water

RNAse-free water (Sigma Aldrich)

Ethanol 96% (Penta)

DNA Isolation

RCLB (Red Cell Lysis Buffer):

320 mM sacharose (Sigma Aldrich)

1% (v/v) Triton X-100 (Sigma Aldrich)

12 mM Tris-HCl pH=7,5 (Sigma Aldrich)

5 mM MgCl2 (SERVA)

WCLB (White Cell Lysis Buffer):

120 mM EDTA pH=8

375 mM NaCl (SERVA)

Proteinase K from *Tritirachium album* 30 units/mg (Sigma Aldrich)

10% SDS (Sigma Aldrich)

6M NaCl (SERVA)

100% ethanol (Sigma Aldrich)

dH2O

RNA Isolation

QIAamp® RNA Blood Mini Kit (QIAGEN)

Buffer EL

Buffer RLT

Buffer RW1

Buffer RPE

Reverse transcription

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

10X RT Buffer

10X RT Random primers

25X dNTP mix (100 mM)

MultiScribe reverse transcriptase (50 U/µL)

HLA Genotypization

Olerup SSP™ DR low resolution (GenoVision)

Olerup SSP™ DQ low resolution (GenoVision)

Olerup SSP™DQB1*02 (GenoVision)

Olerup SSP™DQB1*03 (GenoVision)

Olerup SSP™ DQB1*04 (GenoVision)

Olerup SSP™ DQB1*05 (GenoVision)

Olerup SSP™ DQB1*06 (GenoVision)

Olerup SSP® DQA1 (GenoVision)

Taq DNA polymerase (recombinant) 5U/µl 500U (Fermentas)

Agarose gel electrophoresis

TBE buffer:

890 mM Tris base (Roth)

890 mM boric acid (Amresco)

20 mM EDTA pH=8 (FNKV pharmacy)

dH2O

Agarose (Invitrogen)

GelRed Nucleic Acid Stain (Biotium)

Bromophenol Blue Loading Solution (Promega)

100bp DNA Ladder (Central European Biosystems)

Isolation of blood cells

RBC Lysis Buffer

NH4Cl (Sigma Aldrich)

NaHCO3 (Lachema)

Disodium EDTA (Sigma Aldrich)

dH2O

Buffer B

PBS pH 7.4 (Gibco)

0,1% (w) BSA (Sigma Aldrich)

Buffer E

PBS pH 7.4 (Gibco)

0,1% (w) BSA (Sigma Aldrich)

2 mM EDTA

Dynabeads CD14 (Invitrogen)

Dynabeads CD19 pan B (Invitrogen)

<u>Chromatin Immunoprecipitation</u>

Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore)

ChIP Dilution Buffer

Low Salt Immune Complex Wash Buffer

High Salt Immune Complex Wash Buffer

LiCl Immune Complex Wash Buffer

TE Buffer

Phenylmethanesulfonyl fluoride

Protein A Agarose/Salmon Sperm DNA

Pepstatin A

Aprotinin from bovine lung

5M NaCl

<u>Quantitative PCR</u>

Gene Expression Master Mix (Applied Biosystems)

Primers and probes (IDT)

4.2. Antibodies

Anti-trimethyl-Histone H3(Lys9), clone 6F12-H4 (mouse monoclonal) (Millipore)

Anti-acetyl-Histone H3 (rabbit polyclonal) (Millipore)

Anti-Histone H3, CT, pan, clone A3S (rabbit monoclonal) (Millipore)

Normal Rabbit IgG (Millipore)

4.3. Primer and probe sequences

RNA Expression - Primers and probe for quantitative PCR

Several primer sequences were taken from the paper of Fernandez et al.(2003) , the rest was designed de novo.

*DQA1*01:*

Forward GAAGGAGACTGCCTGGCG

Reverse CATGATGTTCAAGTTGTGTTTTGC

*DQA1*02*:

Forward TTACGGTCCCTCTTGCCAGTT

Reverse TTGCGGGTCAAATCTAAGTCTGT

*DQA1*03*:

Forward GGTCCCTCTGGGCAGTACAG

Reverse CAAATTGCGGGTCAAATCTTCT

*DQA1*04*:

Forward GTACACCCATGAATTTGATGGAGAC

Reverse CAGGATGTTCAAGTTGTGTTTTGTC

*DQA1*05*:

Forward GATGAGCAGTTCTACGTGGACCT

Reverse GTAGAGTTGGAGCGTTTAATCAGAC

DQA1 Total (for all alleles):

Forward TACAGCTCAGAACAGCAACTGC

Reverse CCCACAATGTCTTCACCTCCA

DRA1:

Forward GGACAAAGCCAACCTGGAAA

Reverse AGGACGTTGGGCTCTCTCAG

Probes:

DQA1*01 CCTGCGGGTCAAAACCTCCAAATTTG

DQA1*02,*03 CCACATAGAACTCCTCGTCTCCATCAAATTCAT

DQA1*04,*05 ACTGTCTGGTGTTTGCCTGTTCTCAGACAA

DRA1 CAACTATACTCCGATCACCAATGTACCTCCAGAG

Chromatin Immunoprecipitation - Primers and probe for quantitative PCR

Primer and probe sequences were designed de novo.

*DQA1*01:*

Forward CCCATCCCTCTTGCGACTG

Reverse GGACTTGAGGAATTGTTCTATGAATAA

*DQA1*02*:

Forward AAAAGAAAAATTCCCATCCCTT

Reverse GGACTTGAGGAACTGTTCTATGAAGAG

*DQA1*03*:

Forward ACCCATCCCTCTTGCGAA

Reverse GACTTGAGGAATTGTTCTATGAACAG

*DQA1*04*:

Forward CCCATCCCTCTTGCGACTA

Reverse CACTCAGAGTGGACTTGAGGAAATA

*DQA1*05*:

Forward ATGCCCATCCCTCTTGCC

Reverse AGAGTGGACTTGAGGAAATGTACTG

DQA1 Total (for all alleles):

Forward AAATGCCCATCCCTCTTGC

Reverse CTCTACTCAGAGTGGACTTGAGGAA

Probe:

CAGACATGCACACACCAGAGAAGATTCCAAT

4.4. Instruments

Single-channel pipettes (HTL)

Microwave oven EMS2840 (Electrolux)

Weighing scales Compact 600 (Bosch)

Autoclave DE-23 (Systec)

Centrifuge Z 300 (Hermle)

Centrifuge MPW-51 (Mechanika Precyzyjna)

Centrifuge Mikro 200 (Hettich)

C1000™ Thermal Cycler (Bio-Rad)

Labcycler Gradient (SensoQuest)

Weighing scales Precisa Model 40SM-200A (Precisa)

Biological Safety Cabinet Class II - EuroFlow Series (Clean Air Techniek B.V.)

Vibrating shaker TK3S (Kartell)

KODAK Gel Logic 1500 Imaging System (Carestream)

Power supply CS-300V (Sigma Aldrich)

Owl™ A1 Large Gel System (Thermoscientific)

MultiSUB Horizontal Gel System (Cleaver Scientific)

Biological Thermostat BT 120M (Laboratorní Přístroje Praha)

Premium U410 Upright Freezer (New Brunswick)

Upright Freezer (Whirpool)

Refrigerator (Liebherr)

4.5. Software

Vector NTI AdvanceTM 11

KODAK Molecular Imaging Software Standard Edition v5.0.1.27

SPSS v16.0

7500 Software v2.0.6

5. Methods

5.1. Subject

The subject of this study was composed of 39 voluntary blood donors, including students and employees of Third Faculty of Medicine and Faculty of Science of Charles University, and also family members of type 1 diabetes patients. Gender ration was 14 males to 25 females (35,9 %: 64,1%). Age range was 9 to 66 years, average age was 35 years. The age of two donors is unknown. Blood was sampled together with past histories after getting acquainted approval.

5.2. Isolation of nucleic acids

5.2.1. Isolation of DNA from human blood

Red Cell Lysis Buffer (RCLB): 54,77 g saccharose, 5 ml Triton X-100, 6 ml 1M Tris-HCl pH=7,5, 2,5 ml 1M MgCl2.6H2O, dH2O to 500 ml

White Cell Lysis Buffer (WCLB): 120 ml 0,5M EDTA, 37,5 ml 5M NaCl, dH2O to 500 ml

All centrifugation steps were performed at 18 000 g

1) Transfer 0,5 ml of blood sample to 1,5 ml tube and add 1 ml RCLB and centrifuge for 6 mins

2) Remove the supernatant and wash the pellet with 1 ml of dH2O, centrifuge for 2 mins; repeat washing step once more

3) Add 235 ul dH2O, 80 ul WCLB, 40 ul 10% SDS and 15 ul Protease K to the pellet and resuspend; incubate at 55°C for 30 mins under rolling

4) Cool the sample to room temperature and add 120 ul of 6M NaCl, mix intensively and centrifuge for 6 mins

5) Transfer the supernatant to the new tube and cetrifuge again for 3 mins

6) Transfer the supernatant to the new tube again and add 1 ml of 96% ethanol cooled to -20°C; precipitate the DNA by gentle shaking of the tube and incubate on ice for 20 mins

7) Centrifuge for 3 mins and remove the supernatant

8) Add 1 ml of 70% ethanol and mix for 3 mins; centrifuge for 3 mins and remove the supernatant; let the pellet dry

9) Dissolve the DNA in 100 ul of dH2O and store at -20°C

5.2.2. Isolation of RNA from human blood

Isolation was performed from full human blood using the QIAamp® RNA Blood Mini Kit (QIAGEN). β-mercaptoethanol was added to RLT Buffer and 96-100% ethanol was added to BPE Buffer prior to the isolation.

1) Lyse the erythrocytes by adding 5 volumes of EL Buffer to 1 volume of blood sample. Incubate on ice for 15 mins, vortex briefly each 5 minutes

2) Spin at 400 g for 10 minutes at 4°C; remove supernatant carefully

3) Add 2 volumes of EL Buffer and resuspend the pellet; spin again and remove supernatant

4) Resuspend the pellet with 600 ul of RLT Buffer

5) Transfer the sample to QIAshredder column, centrifuge at 18 000 g for 2 minutes at 4°C

6) Discard the column and add 600 ul 70% ethanol to the filtrate

7) Transfer the sample to QIAamp column; spin at 12 000 g for 15 seconds and discard the collection tube

8) Wash the column with 700 ul of RW1 Buffer and repeat the spinning; discard the collection tube

9) Add 500 ul BPE Buffer and centrifuge at 12 000 g for 15 seconds

10) Add another 500 ul of RPE Buffer and centrifuge at 18 000 g for 3 minutes; discard the collection tube

11) Centrifuge again at 18 000 g for 1 minute

12) Place the column in clean 1,5 ml tube and add 30 ul RNAse-free water on the membrane; spin at 12 000 g for 1 minute

13) Store isolated RNA at -80°C

5.2.3. Determination of the concentration and purity of isolated nucleic acids

The quality and quantity of isolated DNA was ascertained with the help of spectrophotometer. Measurement was performed at wavelenght λ=260 nm, using the A260/A280 and A260/A240 ratios to determine protein and RNA contamination.

5.3. HLA Genotypization

5.3.1. PCR

HLA genotypization of isolated DNA was performed with the help of Olerup HLA SSP kits – Olerup SSP DQA1 and Olerup SSP DR low resolution. The reaction mix was prepared for every sample according to the table 5.1 . 10 ul of the mix was added to each well and the kit was subjected to PCR according to the table 5.2. Afterwards, samples were subjected 2% agarose gel electrophoresis and assessed using the official Olerup interpretation worksheets.

Tab. 5.1 - The composition of PCR reaction mix

Reagent	**Volume**
dH2O	4,92 μl
PCR Mix	3 μl
DNA (c=30 ng/ul)	2 μl
Taq polymerase	0,08 μl

Tab 5.2 - The thermocycler protocol used for genotypization

Step	**Temperature**	**Duration**	**Number of cycle repetitions**
Initial denaturation	94 °C	120 s	
Denaturation	94°C	10 s	10
Annealing and elongation	65°C	60 s	10
Denaturation	94°C	10 s	20
Annealing	61°C	50 s	20
Elongation	72°C	30 s	20

5.3.2. Agarose gel electrophoresis

The horizontal agarose gel electrophoresis in TBE buffer was used to separate and visualize the products of PCR. Samples were loaded into 2% agarose gel containing GelRed intercalation reagent for the purpose of visualization and separated using the electrical current. The voltage was constant, 5V/cm of gel. UV transluminator MUVB 20 (UltraLum, USA) was used for evaluation.

5.4. White cells isolation

5.4.1. Isolation of leukocytes from human blood

RBC Lysis Buffer (10x concentration): 8,02 g NH_4Cl, 0,84 g $NaHCO_3$, 0,37 g EDTA, dH_2O to 100 ml

1) Transfer 3 ml of the blood sample to 50ml tube and add cold RBC Lysis Buffer to full capacity; invert the tube for approximately 10 minutes, until the liquid has clear red color

2) Spin at 250 g for 15 mins at 4°C, with the cetrifuge set at slow braking; remove the supernatant and resuspend the pellet with 10 ml of cold PBS

3) Repeat the cetrifugation as described above and resuspend the pellet with 3 ml of cold PBS

Next step is crosslinking. From this point forward, the procedure is the same for both CD14/CD19 and leukocyte samples.

Cells were counted from 15 ul of the respective samples using the hemocytometer.

5.4.2. CD 14 and CD 19 cells isolation from human blood

Buffer B: 0,5 g BSA, PBS to 500 ml

Buffer E: 0,5 g BSA, 2 ml 0,5M EDTA, PBS to 500 ml

10x Tris-Gly: 0,47 g glycin, 50 ul 1M Tris-Cl (pH 8), dH_2O to 5 ml

10x Tris-Gly with protease inhibitors: 930 ul 10x Tris-Gly, 10 ul aprotinin, 10 ul pepstatin, 50 ul DMSF

SDS Lysis Buffer: 10 ml 10% SDS, 0,37 g EDTA disodium salt dihydrate (M = 372,2 g/mol), 5 ml 1M Tris(pH 8.1), dH2O to 100 ml

A) *Washing of the beads*

1) Resuspend the beads in its vial and move the required amount (18 ul / 1 ml of blood) to new tubes

2) Add the same amount of Buffer B (for CD14 cells) or E (for CD19 cells) and mix - if the amount is lower than 1 ml, add 1 ml instead

3) Place the tubes on magnet for 1 min; carefully remove the supernatant

4) Resuspend with Buffer B/E, with the amount equal to the original amount of the beads; store on ice

Following steps should be performed on ice unless mentioned otherwise; all centrifugation steps occur at 4°C temperature

B) *Removal of free CD14*

1) Make note of the original volume of the blood sample, transfer it to 50ml tube and dilute it with double the amountof Buffer E

2) Centrifuge at 500 g for 10 mins with slow braking on; remove the upper layer of blood plasma carefully in order not to disturb the white cell layer beneath

3) Resuspend with Buffer E to the original volume of the blood sample

C) *Isolation of CD14 cells*

1) Add the CD14 beads washed in Buffer B in required amount (18 ul / 1 ml of blood); incubate at 4°C for 30 mins under rolling

2) Place on magnet for 3 mins; carefully transfer the supernatant to the new tube marked as „CD19“ and place it on magnet again to check whether beads stayed in the original tube, without disturbing the bead pellet on the wall

3) Wash the beads with Buffer B (in approximately the same amount like the original blood sample), place on magnet for 3 mins and remove the supernatant

4) Repeat washing 2-3 more times until the supernatant is clear

5) Resuspend the beads in 3 ml of PBS

D) *Isolation of CD19 cells*

1) Add the CD19 beads to the tube marked as „CD19“, containing the supernatant from the step ahead; incubate at 4°C for 20 mins under rolling

2) Place on magnet for 3 mins; remove the supernatant

3) Wash the beads with Buffer E (in approximately the same amount like the original blood sample), place on magnet for 3 mins and remove the supernatant

4) Repeat washing 2-3 more times until the supernatant is clear

5) Resuspend the beads in 3 ml of PBS

5.5. RNA Expression analysis

5.5.1. Reverse transcription

In order to obtain cDNA necessary for expression analysis, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used according to manufacturers instructions. All steps were performed on ice. The reaction mix for each reaction was prepared according to

table 5.3, 200 ng RNA was added. To reach total sample volume of 20 ul, dH2O was added in necessary quantity. Samples were loaded into thermocycler and subjected to program described in table 5.4 below. After the completion of the program, samples were stored at -20°C.

Tab. 5.3 - The composition of reverse transcription reaction mix

Reagent	**Volume**
dH2O	4,2 µl
10x RT Buffer	2 µl
25x dNTP mix	0,8 µl
10x RT Random primers	2 µl
MultiScribe reverse transcriptase	1 µl

Tab 5.4 - The thermocycler protocol used for reverse transcription

Step	**Temperature**	**Duration**
Hybridization	25 °C	10 min
Reverse transcription	37°C	120 min
Enzyme inactivation	85°C	5 min

5.5.2. Quantitative PCR

To observe the gene product increase in the real time, quantitative PCR was performed. All steps were performed on ice. Before adding, cDNA was diluted 10 times, to the final concentration 30 ng/ul. Reaction mix was prepared according to table 5.5. 7,5 ul of reaction mix was transfered to each well of the plate, all reactions were prepared in triplets. 5 ul of diluted cDNA was added to each well. The plate was centrifuged briefly at 2000 g, then it was subjected to quantitative PCR program according to the table 5.6.

Tab. 5.5 - The composition of quantitative PCR reaction mix

Reagent	**Volume**
Gene Expression Master Mix	6,25 µl
Forward primer (10 uM)	0,375 µl
Reverse primer (10 uM)	0,375 µl
Probe (5 uM)	0,5 µl

Tab 5.6 - The thermocycler protocol used for quantitative PCR

Step	**Temperature**	**Duration**	**Number of cycle repetitions**
UNG activation	50 °C	2 mins	
Polymerase activation	95°C	10 mins	
Denaturation	95°C	15 s	40
Hybridization and elongation	60°C	60 s	40

5.6. Histone modification analysis

5.6.1. Crosslinking

1) Add 81 ul of 37% formaldehyde to the sample and mix gently; incubate at room temperature for 10 mins

2) Add 333 ul of 10x Tris-Gly with protease inhibitors to stop the process, mix and incubate on ice for 3 mins

3) Centrifuge at 250 g for 5 mins; remove the supernatant

4) Wash with 3 ml PBS, followed by centrifugation at 250 g for 5 mins; repeat the washing one more time

5) Resuspend the pellet with SDS Lysis Buffer (48 ul / 1 million cells); divide to aliquotes of 300 ul as a preparation for later sonication

6)Incubate on ice for 10 mins, then freeze at -80°C

5.6.2. Sonication

Samples were processed using Bioruptor, with cycles set for 30 seconds sonication and 30 seconds pause, with constant temperature 4°C. CD14/CD19 cells were subjected to 50 cycles, leukocytes to 5 cycles.

5.6.3. Chromatin Immunoprecipitation

Elution Buffer: 300 ul 0,1M $NaHCO_3$, 300 ul 10% SDS, 2,4 ml dH_2O

1) Centrifuge sample at 13 000 rpm for 10 mins at 4°C and transfer the supernatant to the new tube

2) Dilute the supernatant with ChIP Dilution Buffer with protease inhibitors tenfold

3) Pre-clear the sample by addind Salmon Sperm DNA/Protein A Agarose-50% Slurry to the sample (75 ul per 2 ml of sample) and incubate for 1 h at 4°C with rotation

4) Centrifuge at 2000 g for 1 min and transfer the supernatant to the new tube

5) Divide the supernatant to differently marked tubes according to table 5.7

6) Add the primary antibodies and incubate overnight at 4°C with rotation

7) Add 60 ul of Salmon Sperm DNA/Protein A Agarose Slurry and incubate for 1 h at 4°C with rotation

8) Centrifuge at 500 g for 1 min and carefully remove the supernatant

9) Wash the sample successively with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer and two times with TE Buffer, each time by adding 0,7 ml of respective buffer to the sample, 5 min incubation (at 4°C for first two buffers, at room temperature later) and centrifugation at 500 g for 1 min

10) After last washing step remove the supernatant and add 65 ul of fresh Elution Buffer

11) Incubate at room temperature for 15 mins, mix every 3 minutes

12) Centrifuge at 500 g for 1 min and transfer 50 ul of supernatant to the new tube

13) Add 65 ul of Elution Buffer to the pellet and incubate at 65°C for 15 mins, mix every 5 minutes

14) Centrifuge at maximum speed and transfer 50 ul of supernatant to the tube holding the previously transfered 50 ul of supernatant

15) Reverse the crosslink by adding 5 ul of 5M NaCl and incubating overnight at 65°C

Tab. 5.7 - The sample setup for chromatin immunoprecipitation; each of the samples in the table below is prepared in triplicate. Input samples are immediately frozen at -80°C and are only subjected to decrosslinking and purification, from step 15 onwards.

Anti-histone H3	**Anti-acetyl-histone H3**	**Anti-trimethyl-histone H3**	**Background (IgG)**	**Input**
1 ml	1 ml	1 ml	1 ml	100µl

5.6.4. Purification

Samples were purified by MinElute PCR Purification Kit (QIAGEN) according to manufacturer's protocol. All centrifugation steps were performed at 17 900 g at room temperature. 96% ethanol was added to Buffer PE before use.

1) Add 5 volumes of Buffer PB to 1 volume of sample and mix

2) Transfer the sample to MinElute column and spin for 1 minute; discard flow-through

3) Add 750 ul Buffer PE to the column and spin for 1 minute; discard the flow-through

4) Centrifuge once more for 1 minute to remove residual ethanol

5) Discard the collection tube and place the column in a clean 1,5 ml tube

6) Elute DNA by adding 10 ul dH2O; let the column stand for 1 minute, then centrifuge for 1 minute

7) Discard the column and store eluted DNA at -20°C

5.6.5. Quantitative PCR

In order to evaluate the results of chromatin immunoprecipitation, quantitative PCR was performed, using purified samples. The process is very much similar to the process used when measuring RNA expression. The amplification mix was prepared according to table 5.8. 7,5 ul of the amplification mix was transfered to each well on the plate, 5 ul of DNA sample was then added. The plate was centrifuged briefly at 2000 g before initiating the program according to the table 5.9.

Tab. 5.8 - The composition of quantitative PCR reaction mix

Reagent	**Volume**
Gene Expression Master Mix	6,25 µl
Forward primer (10 uM)	0,375 µl
Reverse primer (10 uM)	0,375 µl
Probe (5 uM)	0,5 µl

Tab 5.9 - The thermocycler protocol used for quantitative PCR

Step	**Temperature**	**Duration**	**Number of cycle repetitions**
UNG activation	50 °C	2 mins	
Polymerase activation	95°C	10 mins	
Denaturation	95°C	15 s	40
Hybridization and elongation	60°C	60 s	40

5.7. Statistical methodology

The results were statistically evaluated with the help of SPSS statistical software, version 16. RNA expression levels were compared using the nonparametrical Mann-Whitney test with 95% confidence interval ($p < 0,05$). The differences between alleles were evaluated using the Wilcoxon signed-rank test. The correlations were performed using both Pearson and Spearman correlation.

6. Results

6.1. HLA Genotypization

The participants of the study were subjected to genotypization of their HLA class II genes DRB1, DQA1 and DQB1. The example of experimental genotypization result can be reviewed on figure 6.1. The frequencies of individual haplotypes are shown in table 6.1. The DRB1*11 – DQA1*0505 – DQB1*0301 haplotype was found to be the most common (19,48%), followed by DRB1*01 – DQA1*0101 – DQB1*0501 and DRB1*07 – DQA1*0201 – DQB1*0202 haplotypes (both 15,58%). The complete list of blood donors and their genotypes can be seen in table 6.2. One homozygotic donor was registered, with haplotype DRB1*15 – DQA1*0102 – DQB1*0602.

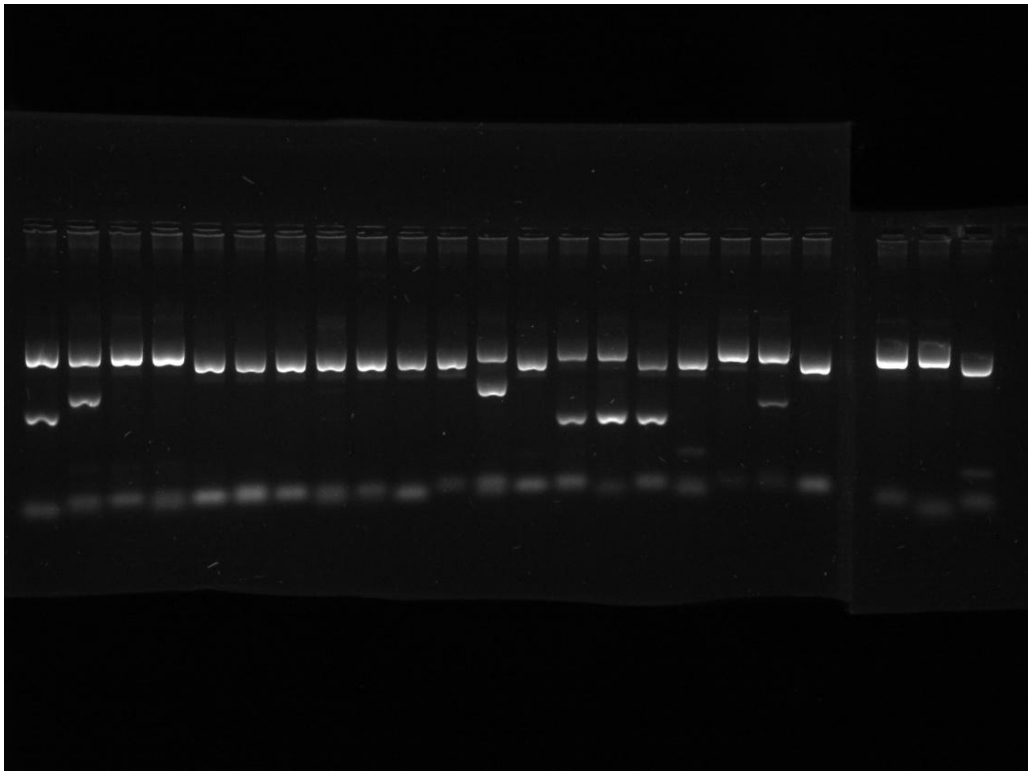


Fig 6.1 – The example of genotypization result. The picture shows the final agarose gel picture for specific DQB1*03 allele, DQB1*0301 specifically. Sample comes from subject number 24. Upper band line presents the positive control, individual bands in lower area of the gel present specific products, whose combination allows us to determine concrete genotype, according to official evaluation worksheet provided by manufacturer.

Tab 6.1 - The haplotypes found in subject group in order according to their frequency

DRB1	DQA1	DQB1	number of carriers	frequency (%)
11	0505	0301	15	19,48
01	0101	0501	12	15,58
07	0201	0202	12	15,58
15	0102	0602	8	10,39
13	0103	0603	5	6,49
03	0501	0201	5	6,49
16	0102	0502	4	5,19
08	0401	0402	3	3,90
13	0505	0301	3	3,90
04	0301	0302	2	2,60
13	0102	0604	1	1,30
01	0505	0301	1	1,30
04	0301	0305	1	1,30
04	0302	0301	1	1,30
07	0201	0303	1	1,30
09	0301	0303	1	1,30
12	0505	0301	1	1,30
13	0102	0603	1	1,30

Tab 6.2 - The list of the subjects who donated blood, with their individual genotypization results

Subject nr.	DRB1	DQA1	DQB1
1	08	0401	0402
	11	0505	0301
2	07	0201	0202
	15	0102	0602
3	07	0201	0202
	12	0505	0301
4	11	0505	0301
	13	0102	0604
5	11	0505	0301
	13	0103	0603
6	01	0101	0501
	01	0505	0301
7	07	0201	0202
	11	0505	0301
8	01	0101	0501
	15	0102	0602
9	01	0101	0501
	11	0505	0301
10	11	0505	0301
	13	0102	0603
11	08	0401	0402
	11	0505	0301
12	04	0301	0305
	07	0201	0202
13	16	0102	0502
	13	0103	0603
14	03	0501	0201
	13	0103	0603
15	01	0101	0501
	03	0501	0201
16	07	0201	0202
	11	0505	0301
17	01	0101	0501
	11	0505	0301
18	11	0505	0301
	07	0201	0202
19	01	0101	0501
	11	0505	0301
20	16	0102	0502
	04	0301	0302

Sample nr.	DRB1	DQA1	DQB1
21	01	0101	0501
	16	0102	0502
22	04	0301	0302
	07	0201	0202
23	01	0101	0501
	11	0505	0301
24	16	0102	0502
	11	0505	0301
25	01	0101	0501
	07	0201	0202
26	07	0201	0202
	08	0401	0402
27	15	0102	0602
	15	0102	0602
28	13	0505	0301
	15	0102	0602
29	13	0505	0301
	15	0102	0602
30	09	0301	0303
	15	0102	0602
31	04	0302	0301
	07	0201	0303
32	11	0505	0301
	15	0102	0602
33	07	0201	0202
	01	0101	0501
34	07	0201	0202
	03	0501	0201
35	03	0501	0201
	13	0505	0301
36	15	0102	0602
	11	0505	0301
37	13	0103	0603
	01	0101	0501
38	07	0201	0202
	13	0103	0603
39	03	0501	0201
	01	0101	0501

6.2. QAP - DQA1 mRNA expression analysis

The expression of DQA1 mRNA was measured relatively, by comparison to the expression levels of HLA DRA1. The subjects with non-standard haplotypes were omitted from the experiment due to uncertainty about their QAP alleles. In total, cDNA from 23 individuals was tested for expression levels. Table 6.3 shows QAP alleles with a known association to HLA haplotypes. These associations were determined by bisulfite sequencing, performed as a part of another study done earlier in our laboratory (Čepek, 2012). QAP allele 1.3b was not present in this subject group and was omitted from further calculations.

Tab 6.3 - Known QAP alleles and the associated haplotypes as determined by bisulfite sequencing study (Čepek, 2012)

DRB1	DQA1	DQB1	QAP
01	0101	0501	1.1
16	0102	0502	1.2K
15	0102	0602	1.2L
13	0103	0603	1.3a
14	0104	0503	1.3b
13	0102	0604,0609	1.4
07	0201	0202,0303	2.1
04	03	03,0202	3.1
11,12	0505	0301	4.1A
03	0501	0201	4.1B
08	0401	0402	4.2

Results were calculated using the Mann-Whitney test. This nonparametrical test was chosen due to being better suited for our data set with low number of individuals and non-normal distribution. Figure 6.2 shows the relative mRNA expression levels for individual QAP alleles (in relation to the DRA1 gene expression levels, which are considered to be a baseline).

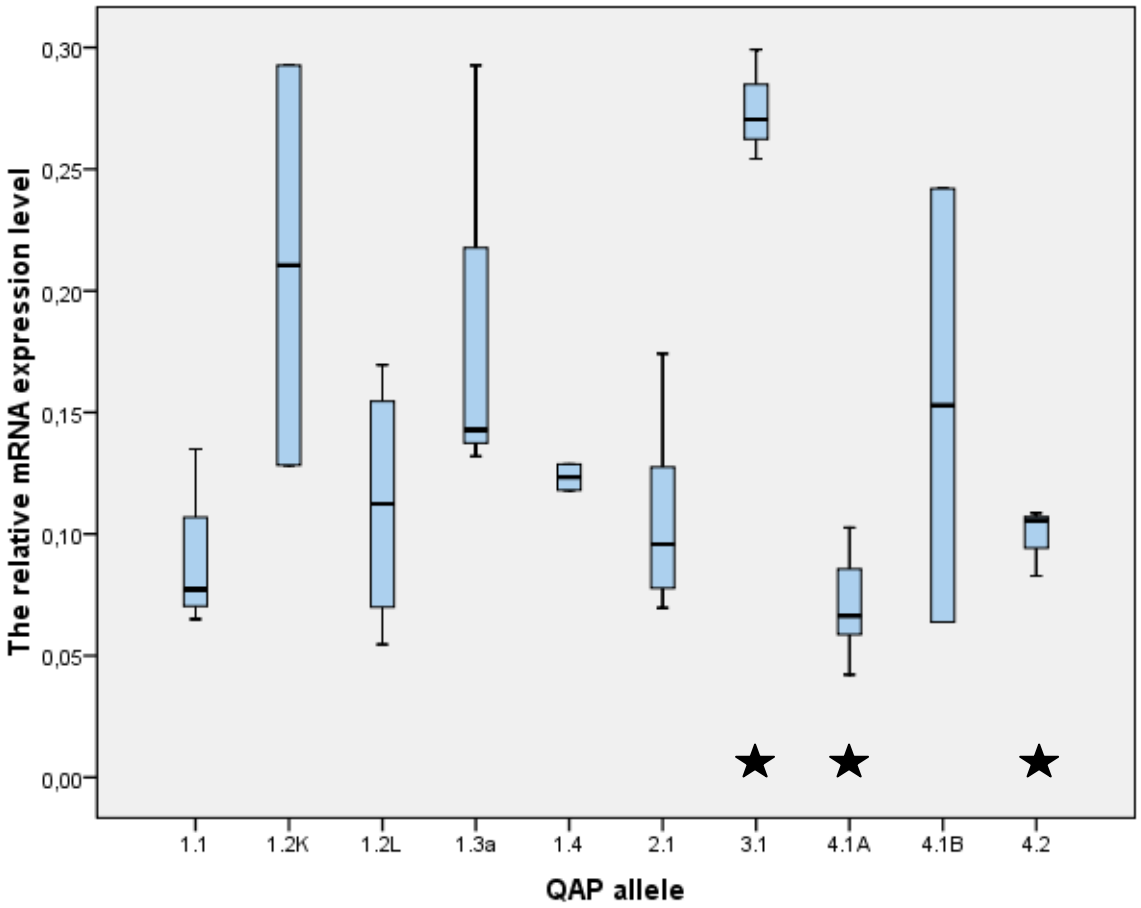


Fig 6.2 – Calculated mRNA expression levels of individual QAP alleles, using DRA1 gene expression as baseline

As seen from the graph, several alleles (marked by star) show significant differences in relative expression level compared to others. This is especially true for QAP allele 4.1A, where the relative expression level is significantly lower (with p-value < 0,05) compared to the most of the other alleles. Alleles 3.1 and 4.2 have also shown differences in relative expression levels that are statistically significant, the relative expression of allele 3.1 being considerably higher than most. Calculated p-values for individual allelic comparisons are listed in table 6.4, with significant values highlighted.

Tab 6.4 - Calculated p values for differences between individual QAP alleles; values with statistical significance ($p < 0,05$) are highlighed with darker background

	1.1	1.2K	1.2L	1.3a	1.4	2.1	3.1	4.1A	4.1B	4.2
1.1		0.165	0.386	0.077	0.355	0.394	0.034	0.317	0.999	0.289
1.2K			0.355	0.767	0.439	0.096	0.564	0.025	0.439	0.083
1.2L				0.289	0.999	0.831	0.034	0.230	0.643	0.724
1.3a					0.083	0.071	0.275	0.008	0.564	0.050
1.4						0.317	0.083	0.025	0.999	0.083
2.1							0.020	0.036	0.999	0.999
3.1								0.008	0.083	0.050
4.1A									0.297	0.038
4.1B										0.999
4.2										

As a last step before moving on to the chromatin immunoprecipitation, we attempted to find correlation between the expression levels of QAP haplotypes and age of subjects. We used both Pearson and Spearman correlation. Spearman correlation showed significant relationship between age and mRNA expression levels for QAP alleles 1.2L, 2.1 and 3.1. Pearson correlation resulted in the same findings, plus also QAP allele 4.1A. Spearman correlation is however better suited for our dataset, as it is nonparametrical and more adequate for small number of values, such as in this case, and we decided to use it for evaluation. Based on calculated correlation coefficients, we established relationship between age and expression levels for QAP haplotypes 1.2L, 2.1 and 3.1 at statistical significance level $p<0,05$.

Tab 6.5 – Results of correlation between age and DQA1 expression for each QAP allele; alleles 1.2K, 1.4 and 4.1B had only two subjects, therefore correlation could not be calculated; values with statistical significance ($p < 0,05$) are highlighted with darker background

QAP allele	N	Pearson correlation		Spearman correlation	
		correlation coefficient	significance	correlation coefficient	significance
1.1	4	0,462	0,538	0,800	0,200
1.2K	2	-	-	-	-
1.2L	4	0,968	0,032	0,949	0,050
1.3a	3	0,834	0,372	0,500	0,667
1.4	2	-	-	-	-
2.1	6	0,954	0,003	0,943	0,005
3.1	3	0,997	0,049	0,999	0,001
4.1A	15	0,532	0,041	0,455	0,088
4.1B	2	-	-	-	-
4.2	3	0,684	0,521	0,500	0,667

6.3. Chromatin immunoprecipitation

As we designed primers for quantitative PCR de novo, it was necessary first to control their specificity. The example of specificity check is shown in figure 6.3.

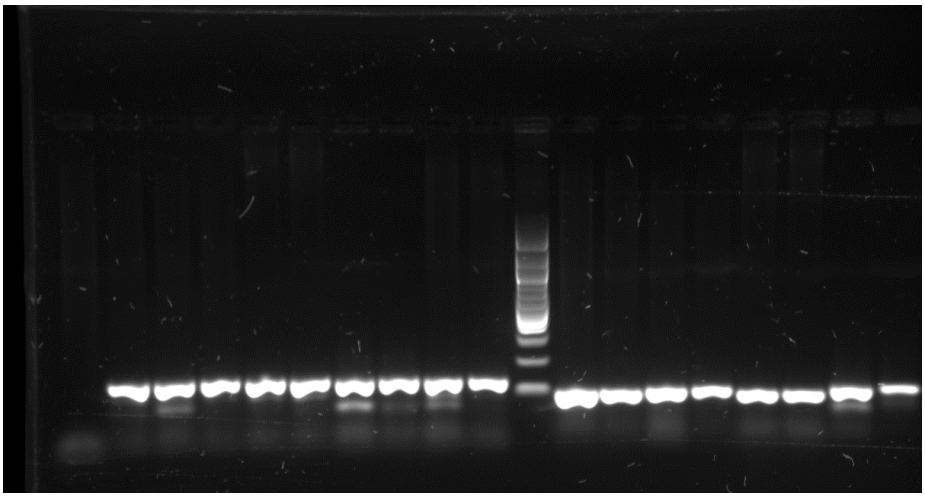


Fig 6.3 - The result of „total" primers verification. Samples (in dublets) come from homozygotic subjects with alleles DQA1*01, *02, *03, heterozygous *04/*05 and homozygous *05 (no DQA1*04 homozygote was available in our dataset). All bands reach the required lenght (slightly over 100bp); sample in first well evaporated during PCR process.
On the right side of the marker there are identical samples (without homozygous *05 sample) in the same order treated with their respective specific primers. This was intended as concentration check, no negative controls are thus included in this case

For chromatin immunoprecipitation, seven leukocyte samples in total have been analyzed, from donors with DQA1 alleles DQA1*01, *02, *03 and *05. These donors were specifically selected from the group of subjects with measured mRNA expression levels. All samples were treated with anti-trimethyl-histone H3 and anti-acetyl-histone H3, for alleles DQA1*01 and *05 the anti-histone H3 antibody was also used. Table 6.6 shows the mean, median and standard deviation values for each allele and antibody, along with their nonspecific background values; these values represent the percentage of input (base sample without any antibodies).

Tab 6.6 – Basic descriptive statistics for histone modifications expressed as the percentage of input; only one sample with allele *02 was measured, therefore no standard deviation can be calculated. Legend: 3M – anti-trimethyl-histone H3, A – anti-acetyl-histone H3, H3 – anti-histone H3, BG - background

	*01 / 3M	*01 / A	*01 / H3	*01 / BG	*02 / 3M	*02 / A	*02 / BG
Mean	0,139	0,236	0,045	0,174	0,511	0,471	0,114
Median	0,083	0,142	0,046	0,087	0,511	0,471	0,114
Std. Deviation	0,145	0,248	0,030	0,258			
	*03 / 3M	*03 / A	*03 / BG	*05 / 3M	*05 / A	*05 / H3	*05 / BG
Mean	0,403	0,364	0,544	0,070	0,181	0,064	0,110
Median	0,403	0,364	0,544	0,079	0,103	0,075	0,092
Std. Deviation	0,422	0,351	0,451	0,031	0,119	0,026	0,070

Figures 6.4A-D show median values for each allele and antibody as comparison to background. It is clearly visible that background values are very high, comparable to specific antibodies. This indicates that only low amount of histone modifications is present at the amplified area of DQA1 promoter region.

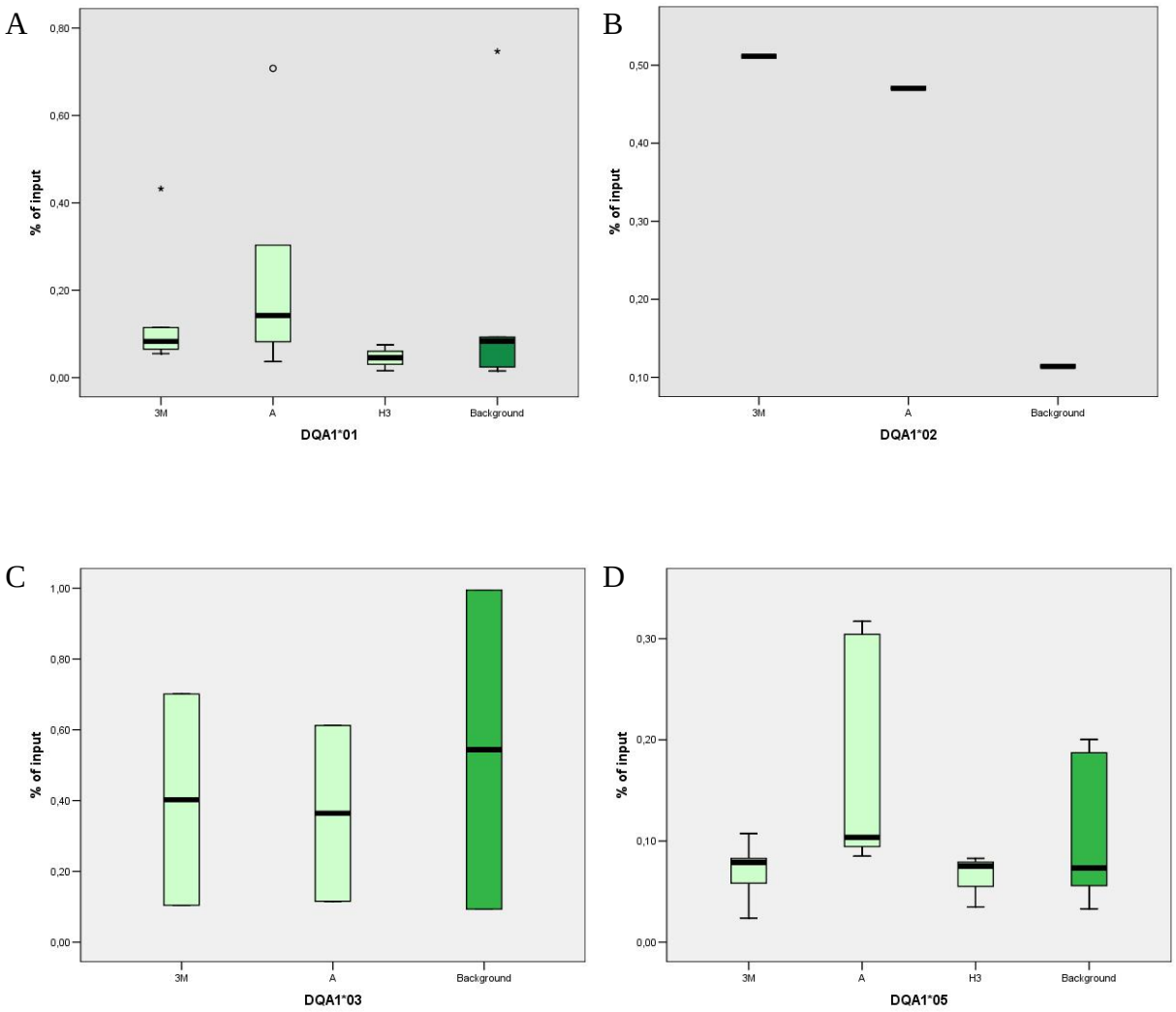


Fig 6.4 - Boxplot showing amount of specific antibody measured, expressed as percentage of input (base sample), for alleles DQA1*01 (**A**), *02 (**B**), *03 (**C**) and *05 (**D**)

In order to determine whether there are significant differences between DQA1 alleles *01 and *05, Wilcoxon signed-rank test was used to compare them. Alleles *02 and *03 could not be calculated due to low number of samples analyzed. Results can be reviewed in table 6.7. No statistically significant difference was found for either of the histone modification variants.

Tab 6.7 – Significance values for the differences between alleles DQA1*01 and *05 for each specific antibody

	*05 / 3M - *01 / 3M	*05 / A - *01 / A	*01 / H3 - *05 / H3
Significance	0,500	0,225	0,109

Next, we tried to find out whether there are significant differences in acetylation and trimethylation levels between QAP alleles. Mann-Whitney test was used to compare QAP alleles 1.1, 1.2K and 1.2L, as those had enough data for statistics. Calculated statistical significances are presented in table 6.8, no significant differences were found.

Tab 6.8 – Significance values for differences in trimethylation and acetylation between selected QAP alleles

01-3M	1.1	1.2K	1.2L
1.1		0.667	0.333
1.2K			0.667
1.2L			

01-A	1.1	1.2K	1.2L
1.1		0.667	0.999
1.2K			0.667
1.2L			

Finally, we looked for possible correlation between histone modification status and mRNA expression levels for DQA1 alleles *01 and *05. Alleles *02 and *03 could not be calculated due to low number of samples. Both Pearson and Spearman correlations were used, the results are presented in table 6.9. The Spearman correlation has not shown any values on a significance level that would prove the relationship between allelic expression and the modification of its histones. The Pearson correlation coefficient demonstrates the relationship on significance level of 0,05 for both trimethylation and acetylation at allele DQA1*01. As in previous case (QAP:age correlation), Spearman correlation is more appropriate, not only because of small dataset, but also because it is resistant to extreme values, which are present in several groups (see figure 6.2A). Therefore, we decided to use the Spearman correlation results to evaluate the significance of the relationships between mRNA expression and histone modifications of DQA1 alleles. As a result, no relationship between histone modification and DQA1 allele can be labeled as statistically significant at the significance level $p<0,05$.

Tab 6.9 – Pearson and Spearman correlations of the mRNA expression and histone modifications for DQA1 alleles

*01 / 3M	Pearson Correlation	,887	Spearman Correlation	-,029
	Significance	,018	Significance	,957
	N	6	N	6
*01 / A	Pearson Correlation	,825	Spearman Correlation	,314
	Significance	,043	Significance	,544
	N	6	N	6
*01 / H3	Pearson Correlation	-,628	Spearman Correlation	-,500
	Significance	,568	Significance	,667
	N	3	N	3
*03 / 3M	Pearson Correlation	1,000	Spearman Correlation	1,000
	Significance	-	Significance	-
	N	2	N	2
*03 / A	Pearson Correlation	1,000	Spearman Correlation	1,000
	Significance	-	Significance	-
	N	2	N	2
*05 / 3M	Pearson Correlation	-,322	Spearman Correlation	-,300
	Significance	,597	Significance	,624
	N	5	N	5
*05 / A	Pearson Correlation	-,412	Spearman Correlation	,100
	Significance	,491	Significance	,873
	N	5	N	5
*05 / H3	Pearson Correlation	-,308	Spearman Correlation	-,500
	Significance	,801	Significance	,667
	N	3	N	3

7. Discussion

MHC class II genes play an important role in the antigen response and their correct regulation is thus vital for organism's health and survival. The regulation sites in the 5'-untranslated region are hosting various epigenetic modifications, which may have a great influence and can undergo significant changes during life. Epigenetic mechanisms like DNA methylation or histone modifications can also have an impact on the risk that certain alleles carry for certain autoimmune diseases, such as type 1 diabetes (Rakyan et al., 2011). This paper is a continuation of previous work done in our research group, both on healthy controls and patients suffering from type 1 diabetes. This project focuses on MHC class II gene DQA1 of healthy donors and its expression on mRNA level. We also attempted to reveal more details about histone modifications present in the promoter region and possible connection to specific DQA1 alleles.

The results of genotypization show the haplotypes DRB1*11 – DQA1*0505 – DQB1*0301 and DRB1*01 – DQA1*0101 – DQB1*0501 to be among the most common haplotypes in our dataset. Klitz et al. (2003) have performed a comprehensive genotypization study and found the haplotypes DRB1*15 – DQA1*0102 – DQB1*0602 and DRB1*03 – DQA1*0501 – DQB1*0201 to be the most common. These haplotypes were found among our samples as well, albeit in lower frequencies. His research was, however, perfomed on European American population sample, it is thus possible these differences in haplotype frequencies are caused by the variability between populations.

The mRNA expression analysis of DQA1 gene yielded results that were evaluated considering QAP promoter allele present. These QAP alleles were not identified by sequencing, rather by known associations with certain MHC haplotypes. Due to strong linkage disequilibrium existing in MHC class II gene regions it was possible to assign QAP alleles with reasonable certainty. Several blood donors from our group possessed non-typical hyplotypes. These were removed from the dataset for further study, as we could not reliably establish their QAP allele without sequencing study. Our results show signicantly increased expression levels for QAP allele 3.1, while the expression level of allele 4.1A is, on the contrary, significantly lower than most. These results are in agreement with the study published five years ago, where expression levels of DQA1*0301 allele were among the highest measured. DQA1*02 was, on the other hand, described to have generally lower activity (Britten,

Mijovic, Barnett, & Kelly, 2009). Our results suggest lower activity of QAP 2.1 haplotype as well, the difference is not statistically significant, though. QAP promoter allele 4.1A, which precedes DQA1*0505 gene, shows low level of mRNA expression, with the statistical significance compared to most QAP alleles. The study comparing expression levels of DQA1 gene in type 1 diabetes patients and healthy controls reached the similar result, noting that DQA1*05 allele has generally low expression, while DQA1*03 allele has the highest (Donner et al., 2002).

The QAP allele can obviously significantly affect the expression of DQA1 gene, however the activity of the promoter itself may be modified by epigenetic regulation mechanisms. DNA methylation, for instance, which can not only cause silencing by itself, but can also support changes in local chromatin structure. The matter is complicated further by the fact that histone modifications can also have an indirect influence, affecting the activity of MHC class II-specific transcription factors CIITA and RFX instead (Masternak, Peyraud, Krawczyk, Barras, & Reith, 2003). Experiments on tumour cells has shown that inhibiting histone deacetylases and thus increasing the levels of acetylation on histones leads to higher expression of MHC class II DR genes (Chou, Khan, Magner, & Tomasi, 2005; Magner et al., 2000). Based on that, we would expect QAP alleles with higher levels of mRNA expression (notably 3.1) to possess histones with higher rate of acetylation. We have, however, not been able to observe any significant relationship between histone modifications present and expression levels. There were no large differences found between different modifications. Background values were, on the other hand, quite high. This leads us to conclusion that there is a possibility there were only very low amounts of histone proteins binded on the amplified sequence. That, coupled with the fact that only small number of subjects was included, means that no generally applicable conclusions can be made. It is difficult to compare our data with known results, as only a small number of researchers are studying an influence of histone modifications on the promoter region of different MHC class II DQA1 alleles.

8. Conclusions

This thesis is a part of larger research with the aim of uncovering the full extent of the influence the epigenetic regulation of MHC class II genes has on autoimmune diseases and type 1 diabetes mellitus in particular. In this study, we established DRB1 – DQA1 – DQB1 genotypes for 39 healthy blood donors. Then we proceeded to measure mRNA expression levels for specific QAP promoter alleles and to determine the presence of histone modifications in the promoter region of DQA1 gene. Statistical significance was found for allele 3.1, its expression levels being noticeably high, which is in accordance with previous studies showing the DQA1*03 allele to be the most actively expressed DQA1 allele. QAP alleles 4.1A and 4.2 have also shown significant differences, especially the expression levels measured for allele 4.1A were lower than most. We have also discovered the correlation between expression levels and age of subject for alleles 1.2L, 2.1 and 3.1. The study of histone modifications has not brought forth any significant results, the relationship between histone status and DQA1 expression thus remains unclear. Neither were we able to determine any differences between specific QAP alleles with certainty.

Gaining deeper knowledge about epigenetic regulation of MHC class II genes on chromatin level will provide valuable insight into the origin, mechanisms and prevention of autoimmune diseases. As this work can be considered a pilot study and was only performed on low number of individuals, further research involving larger number of subjects will be required to obtain statistically significant data. Also, type 1 diabetes patients should be involved, in order to observe the differences in chromatin status. There is little doubt the epigenetic mechanisms play an important role in autoimmune disorders and full comprehension could grant us the ability to predict and, with timely intervention, potentially prevent many cases of currently incurable illnesses.

List of citations

Aravin, A. A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K. F., . . . Hannon, G. J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell, 31*(6), 785-799. doi: 10.1016/j.molcel.2008.09.003

Bakke, O., & Dobberstein, B. (1990). MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell, 63*(4), 707-716.

Ballestar, E. (2011). Epigenetic alterations in autoimmune rheumatic diseases. *Nat Rev Rheumatol, 7*(5), 263-271. doi: 10.1038/nrrheum.2011.16

Ballestar, E., Esteller, M., & Richardson, B. C. (2006). The epigenetic face of systemic lupus erythematosus. *J Immunol, 176*(12), 7143-7147.

Bird, A., Tate, P., Nan, X., Campoy, J., Meehan, R., Cross, S., . . . Macleod, D. (1995). Studies of DNA methylation in animals. *J Cell Sci Suppl, 19*, 37-39.

Bjornsson, H. T., Fallin, M. D., & Feinberg, A. P. (2004). An integrated epigenetic and genetic approach to common human disease. *Trends Genet, 20*(8), 350-358. doi: 10.1016/j.tig.2004.06.009

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. *Int J Immunogenet, 36*(1), 47-57. doi: 10.1111/j.1744-313X.2008.00823.x

Cameron, P. U., Tabarias, H. A., Pulendran, B., Robinson, W., & Dawkins, R. L. (1990). Conservation of the central MHC genome: PFGE mapping and RFLP analysis of complement, HSP70, and TNF genes in the goat. *Immunogenetics, 31*(4), 253-264.

Cerna, M., Kolostova, K., Novota, P., Romzova, M., Cejkova, P., Pinterova, D., . . . Andel, M. (2007). Autoimmune diabetes mellitus with adult onset and type 1 diabetes mellitus in children have different genetic predispositions. *Ann N Y Acad Sci, 1110*, 140-150. doi: 10.1196/annals.1423.016

Chou, S. D., Khan, A. N., Magner, W. J., & Tomasi, T. B. (2005). Histone acetylation regulates the cell type specific CIITA promoters, MHC class II expression and antigen

presentation in tumor cells. *Int Immunol, 17*(11), 1483-1494. doi: 10.1093/intimm/dxh326

Clark, R. J., & Felsenfeld, G. (1971). Structure of chromatin. *Nat New Biol, 229*(4), 101-106.

Clarke, S. G. (2013). Protein methylation at the surface and buried deep: thinking outside the histone box. *Trends Biochem Sci, 38*(5), 243-252. doi: 10.1016/j.tibs.2013.02.004

Comb, M., & Goodman, H. M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. *Nucleic Acids Res, 18*(13), 3975-3982.

Cruz-Tapias, P., Pérez-Fernández, O. M., Rojas-Villarraga, A., Rodríguez-Rodríguez, A., Arango, M. T., & Anaya, J. M. (2012). Shared HLA Class II in Six Autoimmune Diseases in Latin America: A Meta-Analysis. *Autoimmune Dis, 2012*, 569728. doi: 10.1155/2012/569728

Čepek, P. (2012). *Epigenetická regulace genů HLA II. třídy a jejich role u autoimunitních onemocnění.* (Master's Thesis), Faculty of Science, Charles University, Prague.

Davies, J. L., Kawaguchi, Y., Bennett, S. T., Copeman, J. B., Cordell, H. J., Pritchard, L. E., . . . et al. (1994). A genome-wide search for human type 1 diabetes susceptibility genes. *Nature, 371*(6493), 130-136. doi: 10.1038/371130a0

Demaine, A. G., Hibberd, M. L., Mangles, D., & Millward, B. A. (1995). A new marker in the HLA class I region is associated with the age at onset of IDDM. *Diabetologia, 38*(5), 623-628.

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. *Eur J Immunogenet, 29*(4), 321-330.

Dostal, C., Ivanyi, D., Macurova, H., Hana, I., & Strejcek, J. (1977). HLA antigens in systemic lupus erythematosus. *Ann Rheum Dis, 36*(1), 83-85.

Ehrlich, M., Gama-Sosa, M. A., Huang, L. H., Midgett, R. M., Kuo, K. C., McCune, R. A., & Gehrke, C. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res, 10*(8), 2709-2721.

Eickbush, T. H., & Moudrianakis, E. N. (1978). The histone core complex: an octamer assembled by two sets of protein-protein interactions. *Biochemistry, 17*(23), 4955-4964.

Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene, 21*(35), 5427-5440. doi: 10.1038/sj.onc.1205600

Fekih-Mrissa, N., Klai, S., Zaouali, J., Gritli, N., & Mrissa, R. (2013). Association of HLA-DR/DQ polymorphism with myasthenia gravis in Tunisian patients. *Clin Neurol Neurosurg, 115*(1), 32-36. doi: 10.1016/j.clineuro.2012.04.001

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). *Eur J Immunogenet, 30*(2), 141-148.

Gale, E. A. (2002). The rise of childhood type 1 diabetes in the 20th century. *Diabetes, 51*(12), 3353-3361.

Gaspar-Maia, A., Alajem, A., Meshorer, E., & Ramalho-Santos, M. (2011). Open chromatin in pluripotency and reprogramming. *Nat Rev Mol Cell Biol, 12*(1), 36-47. doi: 10.1038/nrm3036

Gervin, K., Vigeland, M. D., Mattingsdal, M., Hammer, M., Nygard, H., Olsen, A. O., . . . Lyle, R. (2012). DNA Methylation and Gene Expression Changes in Monozygotic Twins Discordant for Psoriasis: Identification of Epigenetically Dysregulated Genes. *PLoS Genet, 8*(1). doi: 10.1371/journal.pgen.1002454

Gospodinov, A., & Herceg, Z. (2013). Chromatin structure in double strand break repair. *DNA Repair (Amst), 12*(10), 800-810. doi: 10.1016/j.dnarep.2013.07.006

Gruen, J. R., & Weissman, S. M. (2001). Human MHC class III and IV genes and disease associations. *Front Biosci, 6*, D960-972.

Huber, L. C., Brock, M., Hemmatazad, H., Giger, O. T., Moritz, F., Trenkmann, M., . . . Jungel, A. (2007). Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. *Arthritis Rheum, 56*(4), 1087-1093. doi: 10.1002/art.22512

Javierre, B. M., Fernandez, A. F., Richter, J., Al-Shahrour, F., Martin-Subero, J. I., Rodriguez-Ubreva, J., . . . Ballestar, E. (2010). Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus. *Genome Res, 20*(2), 170-179. doi: 10.1101/gr.100289.109

Kim, M. S., & Polychronakos, C. (2005). Immunogenetics of type 1 diabetes. *Horm Res, 64*(4), 180-188. doi: 10.1159/000089190

Klitz, W., Maiers, M., Spellman, S., Baxter-Lowe, L. A., Schmeckpeper, B., Williams, T. M., & Fernandez-Vina, M. (2003). New HLA haplotype frequency reference standards: high-resolution and large sample typing of HLA DR-DQ haplotypes in a sample of European Americans. *Tissue Antigens, 62*(4), 296-307.

Kuo, M. H., & Allis, C. D. (1998). Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays, 20*(8), 615-626. doi: 10.1002/(sici)1521-1878(199808)20:8<615::aid-bies4>3.0.co;2-h

Lee, J. Y., Wei, S., & Lee, T. H. (2011). Effects of histone acetylation by Piccolo NuA4 on the structure of a nucleosome and the interactions between two nucleosomes. *J Biol Chem, 286*(13), 11099-11109. doi: 10.1074/jbc.M110.192047

Leonhardt, H., Page, A. W., Weier, H. U., & Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell, 71*(5), 865-873.

Lopez de Castro, J. A., Barbosa, J. A., Krangel, M. S., Biro, P. A., & Strominger, J. L. (1985). Structural analysis of the functional sites of class I HLA antigens. *Immunol Rev, 85*, 149-168.

Magner, W. J., Kazim, A. L., Stewart, C., Romano, M. A., Catalano, G., Grande, C., . . . Tomasi, T. B. (2000). Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *J Immunol, 165*(12), 7017-7024.

Majumder, P., & Boss, J. M. (2011). Cohesin regulates MHC class II genes through interactions with MHC class II insulators. *J Immunol, 187*(8), 4236-4244. doi: 10.4049/jimmunol.1100688

Masternak, K., Peyraud, N., Krawczyk, M., Barras, E., & Reith, W. (2003). Chromatin remodeling and extragenic transcription at the MHC class II locus control region. *Nat Immunol, 4*(2), 132-137. doi: 10.1038/ni883

McFarland, B. J., & Beeson, C. (2002). Binding interactions between peptides and proteins of the class II major histocompatibility complex. *Med Res Rev, 22*(2), 168-203.

Mette, M., Aufsatz, W., van der Winden, J., Matzke, M., & Matzke, A. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J, 19*(19), 5194-5201. doi: 10.1093/emboj/19.19.5194

Miao, F., Chen, Z., Zhang, L., Liu, Z., Wu, X., Yuan, Y. C., & Natarajan, R. (2012). Profiles of Epigenetic Histone Post-translational Modifications at Type 1 Diabetes Susceptible Genes*. *J Biol Chem, 287*(20), 16335-16345. doi: 10.1074/jbc.M111.330373

Miao, F., Smith, D. D., Zhang, L., Min, A., Feng, W., & Natarajan, R. (2008). Lymphocytes From Patients With Type 1 Diabetes Display a Distinct Profile of Chromatin Histone H3 Lysine 9 Dimethylation : An Epigenetic Study in Diabetes. *Diabetes, 57*(12), 3189-3198. doi: 10.2337/db08-0645

Nan, X., Campoy, F. J., & Bird, A. (1997). MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell, 88*(4), 471-481.

Nan, X., Meehan, R. R., & Bird, A. (1993). Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res, 21*(21), 4886-4892.

Neefjes, J., Jongsma, M. L., Paul, P., & Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol, 11*(12), 823-836. doi: 10.1038/nri3084

Novota, P., Cejkova, P., Cerna, M., & Andel, M. (2004). [Genetic risk factors in autoimmune diabetes mellitus, their significance and function]. *Cas Lek Cesk, 143*(3), 159-163.

Oka, S., Furukawa, H., Kawasaki, A., Shimada, K., Sugii, S., Hashimoto, A., . . . Tohma, S. (2014). Protective Effect of the HLA-DRB1*13:02 Allele in Japanese Rheumatoid Arthritis Patients. *PLoS One, 9*(6), e99453. doi: 10.1371/journal.pone.0099453

Okano, M., Bell, D. W., Haber, D. A., & Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell, 99*(3), 247-257.

Pal-Bhadra, M., Leibovitch, B. A., Gandhi, S. G., Chikka, M. R., Bhadra, U., Birchler, J. A., & Elgin, S. C. (2004). Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. *Science, 303*(5658), 669-672. doi: 10.1126/science.1092653

Payer, B., Lee, J. T., & Namekawa, S. H. (2011). X-inactivation and X-reactivation: epigenetic hallmarks of mammalian reproduction and pluripotent stem cells. *Hum Genet, 130*(2), 265-280. doi: 10.1007/s00439-011-1024-7

Peters, A. H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., . . . Jenuwein, T. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell, 107*(3), 323-337.

Pirskanen, R., Tiilikainen, A., & Hokkanen, E. (1972). Histocompatibility (HL-A) antigens associated with myasthenia gravis. A preliminary report. *Ann Clin Res, 4*(5), 304-306.

Pugliese, A., & Eisenbarth, G. S. (2004). Type 1 diabetes mellitus of man: genetic susceptibility and resistance. *Adv Exp Med Biol, 552*, 170-203.

Rakyan, V. K., Beyan, H., Down, T. A., Hawa, M. I., Maslau, S., Aden, D., . . . Leslie, R. D. (2011). Identification of Type 1 Diabetes-Associated DNA Methylation Variable Positions That Precede Disease Diagnosis. *PLoS Genet, 7*(9). doi: 10.1371/journal.pgen.1002300

Reich, D. E., Cargill, M., Bolk, S., Ireland, J., Sabeti, P. C., Richter, D. J., . . . Lander, E. S. (2001). Linkage disequilibrium in the human genome. *Nature, 411*(6834), 199-204. doi: 10.1038/35075590

Reilly, C. M., Mishra, N., Miller, J. M., Joshi, D., Ruiz, P., Richon, V. M., . . . Gilkeson, G. S. (2004). Modulation of renal disease in MRL/lpr mice by suberoylanilide hydroxamic acid. *J Immunol, 173*(6), 4171-4178.

Rice, J. C., Briggs, S. D., Ueberheide, B., Barber, C. M., Shabanowitz, J., Hunt, D. F., . . . Allis, C. D. (2003). Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell, 12*(6), 1591-1598.

Roche, P. A., & Cresswell, P. (1990). Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature, 345*(6276), 615-618. doi: 10.1038/345615a0

Rotter, J. I. (1981). The modes of inheritance of insulin-dependent diabetes mellitus or the genetics of IDDM, no longer a nightmare but still a headache. *Am J Hum Genet, 33*(6), 835-851.

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., . . . Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. *Nature, 419*(6905), 407-411. doi: 10.1038/nature01080

Schranz, D. B., & Lernmark, A. (1998). Immunology in diabetes: an update. *Diabetes Metab Rev, 14*(1), 3-29.

Shoenfeld, Y., Tincani, A., & Gershwin, M. E. (2012). Sex gender and autoimmunity. *J Autoimmun, 38*(2-3), J71-73. doi: 10.1016/j.jaut.2011.12.007

Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E., & Zaller, D. M. (1995). Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature, 375*(6534), 802-806. doi: 10.1038/375802a0

Stastny, P. (1978). Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *N Engl J Med, 298*(16), 869-871. doi: 10.1056/nejm197804202981602

Strahl, B. D., & Allis, C. D. (2000). The language of covalent histone modifications. *Nature, 403*(6765), 41-45. doi: 10.1038/47412

Sylvia, T., Samuel, M., Luis, G., & Zuzet, M. (2013). No association of the HLA-DQ alleles with myasthenia gravis in Cuban patients. *Hum Immunol, 74*(8), 983-985. doi: 10.1016/j.humimm.2013.04.027

Takai, D., & Jones, P. A. (2003). The CpG island searcher: a new WWW resource. *In Silico Biol, 3*(3), 235-240.

Tazi, J., & Bird, A. (1990). Alternative chromatin structure at CpG islands. *Cell, 60*(6), 909-920.

van Beek, E. M., Roelandse-Koop, E. A., Vijzelaar, R., Yilmaz, R., van Hoogstraten, I. M., Schreurs, M. W., . . . Kortlandt, W. (2013). A multiplex assay to rapidly exclude HLA-DQ2.5 and HLA-DQ8 expression in patients at risk for celiac disease. *Clin Chem Lab Med, 51*(6), 1191-1198. doi: 10.1515/cclm-2012-0774

van Steensel, B., & Henikoff, S. (2000). Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. *Nat Biotechnol, 18*(4), 424-428. doi: 10.1038/74487

Vieira, M. L., Caillat-Zucman, S., Gajdos, P., Cohen-Kaminsky, S., Casteur, A., & Bach, J. F. (1993). Identification by genomic typing of non-DR3 HLA class II genes associated with myasthenia gravis. *J Neuroimmunol, 47*(2), 115-122.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., & Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science, 297*(5588), 1833-1837. doi: 10.1126/science.1074973

Waddington, C. H. (2012). The epigenotype. 1942. *Int J Epidemiol, 41*(1), 10-13. doi: 10.1093/ije/dyr184

Weber, M., Hellmann, I., Stadler, M. B., Ramos, L., Paabo, S., Rebhan, M., & Schubeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet, 39*(4), 457-466. doi: 10.1038/ng1990