Molecular Immunology 63 (2015) 505–512

Contents lists available at ScienceDirect

Molecular Immunology

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

Characterization and genotyping of the *DRB1* gene of the major histocompatibility complex (MHC) in the *Marmota monax*, animal model of hepatitis B

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 10 October 2014
Accepted 11 October 2014
Available online 29 October 2014

Keywords:
DRB1 gene
Major histocompatibility complex
Duplication
Antigen binding site
Positive selection
Hepatitis B virus

ABSTRACT

The major histocompatibility complex (MHC)-containing genes are among the most polymorphic in vertebrates. MHC genes code for proteins that are critical in the immune system response. In this study, the polymorphism of the second exon of the MHC class II *DRB* gene was characterized in the Eastern woodchuck (*Marmota monax*). Woodchucks chronically infected with the woodchuck hepatitis virus (WHV) represent the best available animal model for the study of chronic hepatitis B infection in humans. In the genotyped animals we found fifteen alleles, which were expressed in two independent loci and that were named *DRB1A* and *DRB1B* in this work. The 15 alleles investigated showed an elevated divergence. A significant excess of non-synonymous substitutions was detected, which could indicate that a historical positive selection is acting in the woodchuck *DRB1* genes. This hypothesis was confirmed in our study by the high variability in or near the antigen binding sites (ABS) and by the results obtained in sequence variability analyses. This analysis identified the presence of a microsatellite sequence that is located at the start of the second intron, which could further allow the development of a fast and cheap semiautomatic sequencing method.

1. Introduction

Chronic hepatitis in humans is a disease caused by infection with the hepatitis B virus (HBV). It is estimated that 350 million people worldwide suffer from chronic HBV infection and that approximately 1 million people die annually because of HBV-induced liver cirrhosis and/or hepatocellular carcinoma (Dandri and Locarnini, 2012). Thus, despite the existence of an effective HBV vaccine, chronic HBV infection remains a major health problem. Epidemiologic data demonstrate that HBV is a risk factor in liver cancer development but little is known about the molecular mechanisms (Dandri and Locarnini, 2012). Although the virus is not directly cytopathic, liver injuries are produced due to the repeated attempts of the host's immune response to control the infection (Dandri and Locarnini, 2012). Furthermore, experimental evidence has shown that various receptors encoded by the major histocompatibility complex (MHC) play an important role in the antigen presentation to cytotoxic T cells, which are decisive for the HBV infected hepatocytes destruction (Zhou et al., 2002).

The Eastern woodchuck (*Marmota monax*), is a rodent species that is used as a surrogate animal model for research on HBV infection, including disease development, and prevention and therapy of HBV (Menne and Cote, 2007). Natural infection of woodchucks with woodchuck hepatitis virus (WHV) infection causes liver disease highly similar to that induced by HBV infection in humans thereby representing the closest pathobiological model for HBV.(Zhou et al., 2002).

Major histocompatibility complex (MHC) is a genomic region present in all vertebrates and the genes located therein play an important role in antigen presentation and promotion of

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http://dx.doi.org/10.1016/j.molimm.2014.10.011
0161-5890/

T-cell-mediated specific immune response (Kelley et al., 2005). Genes in MHC are classified in three classes, from which Class I genes are involved in intracellular pathogens response while Class II genes encode proteins that recognize extracellular pathogens (Janeway et al., 2004).

The MHC genes are among the most polymorphic genes in vertebrates and the majority of this gene variability is located at residues that code for the antigen binding sites (ABS) (Babik et al., 2005; Babik, 2010). In these sites, a higher rate of non-synonymous substitutions ($d_N/d_S > 1$) is observed, which indicates a positive selection for amino acid substitutions (Bernatchez and Landry, 2003). Pathogens also play a considerable role in MHC diversity because heterozygous animals are favored to gain resistance to pathogens. The heterozygote advantage hypothesis presumes that heterozygous individuals are able to recognize a wider range of antigens (Babik, 2010; Froeschke and Sommer, 2005).

Only a few studies on MHC characterization have been performed in rodents, and most of them have focused on the Critetidae and Muridae families (Froeschke and Sommer, 2005; Oliver and Piertney, 2006). In model organisms such as *Mus musculus, Rattus rattus* and *Peromyscus maniculatus*, a single copy of the *DQB* gene and one or two copies of the *DRB* gene have been described. Aside from the Muridae family, the only complete *DRB* sequence available/described corresponds to Abert's squirrel (*Sciurus aberti*) (Wettstein and colleges, unpublished results, available in GenBank, M97615.1). Partial sequences of the *DRB* gene are also available for members of the Sciuridae (Kuduk et al., 2012), Castoridae (Babik et al., 2005) and Ctenomydae families (Cruterra and Lacey, 2007).

The *DRB* gene of the Alpine woodchuck shows a strong historical positive selection and seems to have undergone duplication events that are indicative of a developing mechanism for strengthening the immune response to fight pathogens (Kuduk et al., 2012). The Alpine woodchuck (*Marmota marmota*) is the closest relative to the animals used in this study. Thus, similar results are expected for *M. monax*.

Due to the evolutionary dynamics of MHC duplications, recombinations and pseudogenes are common which complicates genotyping as they change the gene structure (Babik, 2010).

Thus, MHC genotyping in rodents is still in its infancy when compared to the Muridae family.

All these observations described above indicate that detection and quantification of *Mhc-DRB* variation in the Eastern woodchuck would be very interesting and informative as it could help to improve our understanding of HBV infection and disease development.

Thus, the main purpose of the present study was the characterization of *DRB* gene alleles in the Eastern woodchuck (*M. monax*) as a preliminary step toward the development and optimization of a semiautomatic typing system based on bioinformatics procedures for *DRB* antigens in this species.

2. Materials and methods

2.1. Samples and DNA isolation

A total of 36 woodchuck blood samples from the CIMA research center (Universidad de Navarra) and the Georgetown University Medical Center were used in the present study. Most woodchucks were related to each other (i.e., dams or sires and their offspring) but several unrelated animals were included that were considered as being representative of the woodchuck population. In addition, two pairs of mother-daughter samples were further included to confirm the genotyping suitability. Erythrocytes were removed from blood and leucocytes concentrated prior DNA extraction. Genomic DNA was extracted from blood and liver samples by using a standard phenol–choloroform protocol. DNA concentration was measured with NanoDrop Spectrophotometer (Thermo Scientific Inc.) and samples were stored at −20 °C until use. Woodchucks were treated according to the rules of the Ethical Committee for Animal Testing of the University of Navarra and the Institutional Animal Care and Use committee of Georgetown University.

2.2. Polymerase chain reaction (PCR)

As the Canadian woodchuck is not a model organism, *DRB1* primers from different species were selected from the literature and tested in order to find the best pair for amplifying the second exon of *DRB1* gene (Table 1).

The PCR reaction was performed using a Verity thermocycler (Applied Biosystems, Foster City, Ca). Different temperatures and Mg^{2+} concentrations were tested in order to optimize the conditions for the PCR reaction. In a total volume of 50 μL, 2.5 nM of $MgCl_2$ in 1× buffer, 0.6 nM dNTP, 0.6 μL of GH46_F and GH50_R primers, 0.5U of *Taq* polymerase (Teknovas) and 100ng DNA were added. The cycling scheme was: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 3 min.

PCR products were separated on agarose gels and then purified with the QIAquickPCR Purification Kit (QIAGen, Hilden, Germany) prior to sequencing.

2.3. Cloning

General sequencing techniques do not allow the identification of heterozygous alleles on polymorphic genes as observed for the MHC-*DRB1* gene. To overcome this problem, the PCR products obtained from individual woodchucks were cloned. PCR products from 4 animals were chosen and cloned using the TOPOTA cloning kit (Invitrogen, Carlsbad, CA). Bacterial cells were grown for 24 h at 37 °C and five colonies were selected from each sample for sequencing.

2.4. Sequencing

PCR products were sequenced in an ABIPRISM 3130xl sequencer (Applied Biosystems, Foster City, CA) using the sequencing-kit BigDye v.3.1 (Applied Biosystems, Foster City, CA) and the GTP-BigDye kit (Applied Biosystems, Foster City CA) in case of difficult sequences. Sequencing was performed at the DNA Sequencing and Genotyping Unit of the University of Basque Country (UPV/EHU), which belongs to the General Research Services (SGIKER).

2.5. Bioinformatics analysis

2.5.1. Sequence alignment and allele detection

Sequences were edited with the BioEdit software 7.0.5.3 (Hall, 1999) and single nucleotide polymorphisms (SNPs) were identified visually. For the detection of alleles in heterozygous samples, the Aurkisek 2.0 script (Arrieta-Aguirre et al., 2006) was used, which was previously developed by our group and works on HAPLOFINDER (Miltiadou et al., 2003) that assigns alleles to diploid sequences. Thus, a database was generated containing alleles previously cloned. With this software, every possible combination of the included alleles is calculated, and with these combinations new alleles are inferred from the diploid sequences. As Klein et al. (1990) already proposed, the obtained sequences were named *MhcMamo-DRB1* (from *M. monax*) but in this paper, we used the simplified name *Mamo-DRB1*.