

## NKR genes region and their genetic diversity in horses

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**Abstract:** The genes of the immune response represent a functionally important region of vertebrate genome with demonstrated intense effect of selection. Natural killer cells (NK) are related to antigen recognition process through their highly variable receptors. The genetic variability and different expression of genes for receptors underlies functional variability of individual NK cells. As like in mouse model, the Ly49 receptors on horse NK cells are believed to bind to MHC class I molecules of target cells. The six *Ly49* genes form a gene family located on horse chromosome 6 between 38 200 Kbp – 38 500 Kbp. In this work were identified and genotyped nine microsatellites located in NK cell receptors (NKR) region, which includes genes *Ly49*. Using this methodological work may contribute to the estimate of its genetic diversity in this functionally important region. This methodology will be used for the association analysis of selected diseases in horses.

**Key-Words:** horse, NK cell, NKR, Ly49, genetic diversity, microsatellite

### Introduction

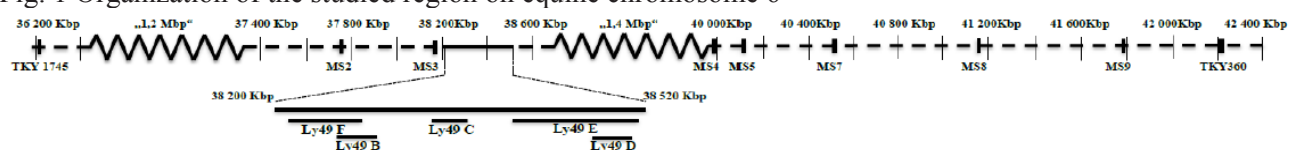
Natural killer (NK) cells mediate important functions in innate resistance to pathogens, including direct cytotoxicity of infected cells and secretion of cytokines [1]. NK cells, a subset of lymphocytes, are part of the first line of defence of the innate immune system. They play a crucial role in cell-mediated immune responses in host defence against pathogens and in tumour cell surveillance [2]. The activities of NK cells are regulated by a diverse spectrum of activating and inhibitory receptors, belonging to both immunoglobulin-related (KIR) and lectin-like receptor (Ly49) families and that bind to cognate ligands [1, 3].

NK receptor gene complexes are intimately associated, both genetically and functionally, with major histocompatibility complex (MHC)

recognition, and interactions of different combinations of NK receptors and MHC class I molecules may contribute significantly to selection and disease resistance [4]. Radiation hybrid mapping and fluorescence in situ hybridization localized horse *Ly49* genes to chromosomes 6q13 (see Fig. 1) [5].

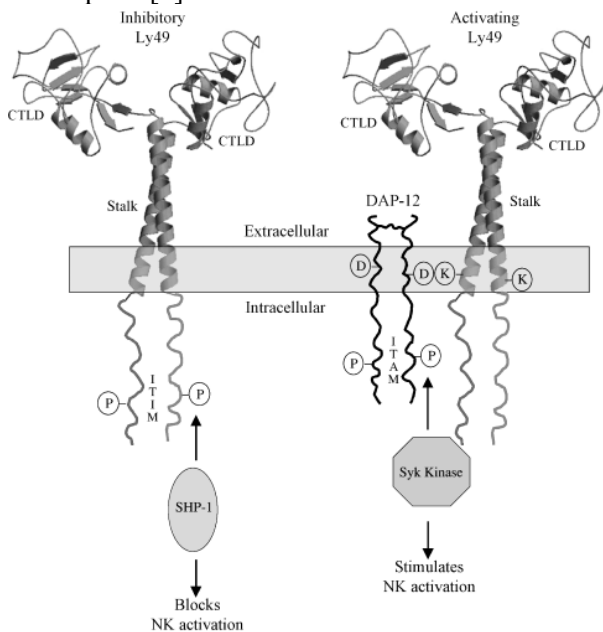
Ly49 receptors are lectin-like type II transmembrane disulfide-bonded homodimers expressed on NK cells and some T-cell subsets and are best known for their role in the regulation of NK cell functions. Cell-mediated cytotoxicity and release of cytokines/chemokines are functions regulated by Ly49 recognition of proteins class I MHC or virus-encoded MHC-like product(s) [1, 6].

Fig. 1 Organization of the studied region on equine chromosome 6



Ly49 expression begins early during NK cell development in the bone marrow (BM) [7]. Activating and inhibitor Ly49 receptors are described as being expressed on both the developing and mature NK cells [2, 6]. The inhibitory Ly49 receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their cytoplasmic tails that become phosphorylated on tyrosine upon receptor engagement (Fig. 1) [8]. The activating Ly49 receptors lack ITIMs, and instead have a positively charged residue in their transmembrane segment that facilitates the association of DAP12 a signalling adapter protein containing immunoreceptor tyrosine-based activating motifs (ITAMs) (Fig. 2) [9, 10].

Fig. 2 Inhibitory and activating Ly49 NK cell receptors with their intracellular signalling counterparts [2].



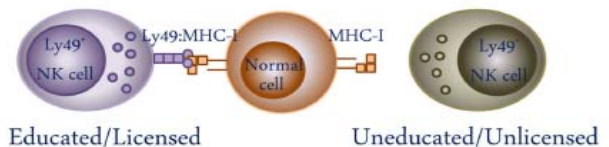
The inhibitory Ly49 receptors are involved in NK cell education, a process in which NK cells acquire function and tolerance toward cells that express “self-MHC-I.” On the other hand, the activating Ly49 receptors recognize altered cells expressing activating ligands (Fig. 3) [6]. Inhibitory Ly49 receptors are generally agreed to be important for the prevention of autoimmunity by suppressing NK cell activation [11]. The activating Ly49 receptors recognize ligands that are expressed on abnormal or infected cells and activate cytokine production and cellular cytotoxicity by NK cells [6].

Domestic mammals represent suitable models for evolutionary biology in general. Among them, the family Equidae consisting of a single genus, *Equus* with different free-living and domesticated species exposed to a variety of pathogens in different habitats

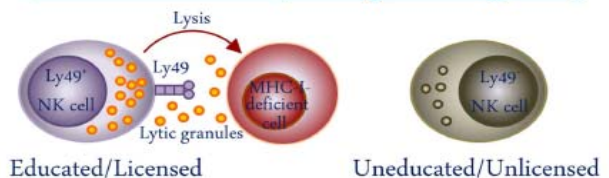
is a suitable model for analyzing diversity and evolution of immunity-related genes. It is a rapidly evolving mammalian family, both at the karyotype and molecular level. Therefore, the Equidae might also be interesting models for studying evolution of NKR genes [12].

Fig. 3 Schematic representation of the role of Ly49 receptors in NK cell development and function.

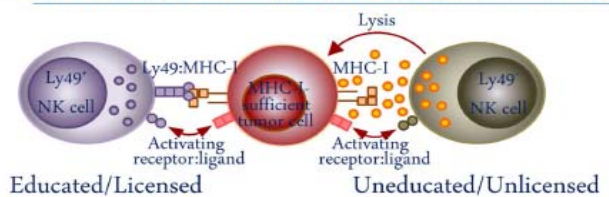
#### A Education and licensing of developing NK cells



#### B Immunosurveillance (missing-self recognition)



#### C Immunosurveillance of MHC-I-sufficient tumors



Legend: (A) During NK cell development, interactions between the inhibitory Ly49 receptors and their self MHC-I ligands on normal cells result in NK cell functional maturation (education/licensing). (B) Licensed Ly49C but not unlicensed Ly49 NK cells recognize MHC-I-deficient cells and kill them through release of lytic granules (missing-self recognition). (C) Tumor cells express ligands which are recognized by activating receptors on NK cells. However, MHC-I-expressing tumor cells can inhibit licensed NK cells through interactions with their inhibitory Ly49 receptors. Unlicensed NK cells will not be inhibited in this way because they lack Ly49 receptors [3].

The aim of this work is to design genetic markers (microsatellites) to study the genetic diversity of NKR region. Selected microsatellites will also be used to describe the genetic variability NKR region in selected populations and for association analysis of selected diseases in horses.

## Material and Methods

### Genotyping animals and their DNA

In this study were genotyped 350 individuals from nine populations of different horse breeds (Marajo,

Campolina, Mangalarga Marchador, Galiceno, Camargue, Romanian horse, Gotland, Yakut and Island horse). Samples of isolated DNA were provided from DNA bank of professor Hořín, Department of Animal Genetics, VFU Brno.

### Selection of microsatellite markers

The whole genome sequences of six horses [13] in the areas of *Ly49* gene family and adjacent parts (35 – 43 Mbp) were used to select markers *in silico*. Suitable panel of microsatellites for the study of *Ly49* region was selected *in silico* by the number of repeats in available horse whole genome sequences. Only microsatellites with the highest number of alleles were selected.

### Design of primers for selected markers

For such markers, primers were designed using the OLIGO software v4.0 (National Biosciences, Inc.; Plymouth, Minnesota).

### Fragmentation analysis for selection of microsatellites

Nineteen markers were designed and tested using a fragment analysis with fluorescently labeled nucleotides (fdCTP) on the panel of horse breeds (Hucul, Czech Warmblood, Danish Warmblood, Quarterhorse, American Miniature Horse, Andalusian horse and Camargue).

Nine markers that showed the highest variability in the test panel of animals were selected. This set of markers was subsequently tested using fluorescent fragment analysis on genetic analyzer ABI PRISM 3500 (Life Technologies Corp.; Carlsbad, USA). The obtained data were analysed in GeneMapper software v4.1 (Life Technologies Corp.; Carlsbad, USA) indicators given in Table 1.

Table 1 Microsatellite markers used in present study.

Marker	Repetition	Range of amplicon (bp)	Number of identified alleles
TKY360	(TG) <sub>n</sub>	288 – 298	6
TKY1745	(TG) <sub>n</sub>	169 – 193	12
Ly49_2	(CA) <sub>n</sub>	267 – 273	4
Ly49_3	(TG) <sub>n</sub>	212 – 222	6
Ly49_4	(CA) <sub>n</sub>	222 – 244	9
Ly49_5	(GT) <sub>n</sub>	330 – 364	14
Ly49_7	(TG) <sub>n</sub>	151 – 165	8
Ly49_8	(TG) <sub>n</sub>	161 – 167	4
Ly49_9	(TG) <sub>n</sub>	257 – 271	8

### Results and Discussion

Suitable panel of microsatellites for the study of *Ly49* region was selected *in silico* by the number of repeats

in available horse whole genome sequences. Only microsatellites with the highest number of alleles were selected. In *Ly49* genes, none of the repetitive sequences was polymorphic. Therefore the study area was expanded to 2 Mbp before and 4 Mbp after *Ly49* gene family to reach the known and described microsatellite markers TKY360 and TKY1745[14].

Markers showing a small number of alleles in the test panel of horse breeds were discarded. The numbers of alleles found in microsatellite markers (Table 1) were identified in populations of fifteen horse breeds from different parts of the world, where 400 individuals were included. Genetic diversity of the *Ly49* region will be estimated based on allele frequencies of the selected microsatellites.

### Conclusion

This work extends the number of genetic markers for analysis of *Ly49* NK cell receptors genetic variability. Previously described alleles of *Ly49* genes [5] could be partly characterized by single nucleotide polymorphisms (SNPs) analysis of this area. We enriched the set of usable markers with 9 microsatellites. The combined genotyping of SNPs and microsatellites may help to define haplotypes of *Ly49* genes. Haplotypes may be more informative for describing the genetic variability in this functionally significant and important part of the immune system.

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### References:

- [1] Kane KP, Lavender KJ, Ma BJ, Ly-49 receptors and their functions, *Critical Reviews in Immunology*, Vol.24, No.5, 2004, pp. 321-348.
- [2] Dimasi N, Biassoni R, Structural and function aspects of the *Ly49* natural killer cell receptors, *Immunology and Cell Biology*, Vol.83, No.1, 2005, pp. 1-8.
- [3] Chan CJ, Smyth MJ, Martinet L, Molecular mechanism of natural killer cell activation in response to cellular stress, *Cell Death and Differentiation*, Vol.21, No.1, 2014, pp. 5-14.
- [4] Kelley J, Walter L, Trowsdale J, Comparative genomics of natural killer cell receptor gene clusters, *PLOS Genetics*, Vol.1, No.2, 2005, pp. 129-139.
- [5] Takahashi T, et al., Natural killer cell receptors in the horse: evidence for the existence of multiple transcribed *LY49* genes, *European*

- Journal of Immunology*, Vol.34, No.3, 2004, pp. 773-784.
- [6] Rahim MMA, et al., Ly49 receptors: innate and adaptive immune paradigms, *Frontiers in Immunology*, Vol.5, No.145, 2014, pp. 1-11.
- [7] Kim S, et al., *In vivo* developmental stages in murine natural killer cell maturation, Vol.3, No.6, 2002, pp. 523-528.
- [8] Nakamura MC, et al., Mouse Ly-49A interrupts early signalling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase, *Journal of Experimental Medicine*, Vol.185, No.4, 1997, pp. 673-684.
- [9] Smith KM, et al., Cutting edge: Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors, *Journal of Immunology*, Vol.161, No.1, 1998, pp. 7-10.
- [10] Tomasello E, et al., Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12, *Journal of Biological Chemistry*, Vol.273, No.51, 1998, pp. 34115-34119.
- [11] Kim S, et al., Licensing of natural killer cells by host histocompatibility complex class I molecules, *Nature*, Vol.436, No.7051, 2005, pp. 709-713.
- [12] Futas J, Horin P, Natural killer cell receptor genes in the family *equidae*: not only Ly49, *PLOS One*, Vol.8, No.5, 2013, pp. 1-11.
- [13] Orlando L, et al., Recalibrating *Equus* evolution using the genome sequence of an early Middle Pleistocene horse, *Nature*, Vol.499, No.7456, 2013, pp. 74-78.
- [14] Tozaki T, et al., Improved resolution of the comparative horse-human map: investigating markers with *in silico* and linkage mapping approaches, *Gene*, Vol.392, No.1-2, 2007, pp. 181-186