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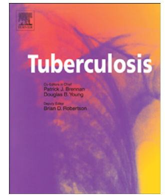

HOST GENETICS OF SUSCEPTIBILITY

Identification of SP110 in horse (*Equus caballus*): Isolation of novel splice variants and evidence of activation effects on macrophages

Qi Chen [1], Qi Tong [1], Hengtao Ge, Wenzhong Li, Jun Liu, Yongsheng Wang, Zekun Guo, Fusheng Quan, Yong Zhang [*]

Key Laboratory of Animal Biotechnology of the Ministry of Agriculture, College of Veterinary Medicine, Northwest A&F University, Yangling 712100, Shaanxi, China

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SUMMARY

SP110 has previously shown to be a genetic determinant of host resistance to the intracellular pathogen infection in mouse and human. However, its relevant biological information in large non-primate animals still remains unknown. Here we report the novel discovery and characterization of three transcript variants of horse SP110. The transcript variant 1 (Tv1) of horse SP110 with the longest open reading frame has four domains (Sp100, SAND, PHD and Bromo domain). Tv2 and Tv3 share the same N-terminal sequence as Tv1, which contains Sp100 and SAND. We show that Tv2 is generated from alternative splicing and deletion of Exon17-Exon18 segment, while Tv3 is generated by pre-mature transcriptional termination at Exon 16. Furthermore, we demonstrate that the heterologous expression of horse SP110 variants stimulate macrophages into an activation-like phenotype. The macrophages underwent a shift in enhancing the secretion of cytokines (interleukin-1 (IL-1) and TNF-α) and accelerating inducible nitric oxide synthase (iNOS) activity, and eventually went into apoptotic cell death. Intriguingly, horse SP110 Tv1 showed more capability to trigger the immune activities compared to Tv2 and Tv3. To our knowledge, the identification of SP110 transcript variants from horse is the first report on biological function of SP110 in perissodactyla animals.

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1. Introduction

The horse (*Equus caballus*), belonging to the taxonomic order Perissodactyla, has evolved over the past 50 million years from a small multi-toed creature into the large, single-toed animal as of today [1]. In worldwide, horses are used for leisure activities, sports and transportation, which makes a great promotion for human civilization. As a comparatively hardy kind of animal, horses were considered to be naturally highly resistant to *Mycobacterium tuberculosis* (*MTB*) infections [2–5], although rare tuberculosis infection cases in horses have been reported [6–8]. For a better understanding of the horse immune system during infection, the availability of species specific reagents for analyzing the major host immune regulatory proteins are essential.

The nuclear body (also known as nuclear domain 10, promyelocytic leukemia protein [PML] oncogenic domain) is a cellular structure that appears to be involved in the pathogenesis of a variety of diseases including acute promyelocytic leukemia and acute viral infections [9,10]. Originally identified in human peripheral blood leucocytes and spleen, SP110, a leukocyte-specific nuclear body component, is thought to play a role in the differentiation of myeloid cells and also act as a transcriptional coactivator [10,11]. The short isoform of human SP110, SP110b, was further found to be a corepressor in retinoid signaling which is involved in the process of a cytoplasmic viral protein regulating host cell transcription [12]. Besides, mutations in SP110 have been reported to have high-penetrance in hepatic veno-occlusive disease [13,14]. Studies of the association between SP110 polymorphisms and the genetic susceptibility to tuberculosis give somewhat contradictory results depending on the populations investigated, i.e. negative correlation in Indonesians [15] but positive relationship in West Africans and Chinese [16,17]. Polymorphism research of bovine SP110 also revealed the genetic relevance between SP110 and paratuberculosis [18]. In 2005, in a previously mapped genetic locus on mouse chromosome 1, designated sst1 (for supersusceptibility to tuberculosis), SP110 homologue gene was identified and proven to be a genetic determinant of host susceptibility to infection of

* Corresponding author. Fax: +86 29 87080085.
E-mail address: zhyonglab@gmail.com (Y. Zhang).
[1] These authors contributed equally to this work.

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intracellular pathogens in mouse. Expression of the SP110 transgene in the sst1 susceptible macrophages limited multiplication not only of *MTB* but also *Listeria monocytogenes* and switched a cell death pathway of the infected macrophages from necrosis to apoptosis [19]. This line of evidence put forward to establish that SP110 plays a major role in the outcome of tuberculosis infection in different species.

Horses are relatively high resistant to *MTB* infection [4,5], which prompted us to explore the potential role of SP110 in the resistance to *MTB* infection in horse. Hitherto, the identification of horse SP110 variants and their potential effects on the host immune capability remains to be addressed.

In this study, we cloned and characterized the full-length horse SP110 gene and its three transcript variants. We further demonstrated, in vitro, that the horse SP110 variants differentially regulate macrophages cytokines (IL-1β, TNF-α and IL-10) and antimicrobial protein (inducible nitric oxide synthase, iNOS) expression and apoptosis.

2. Materials and methods

2.1. Ethics statement

Blood sampling procedure was approved by the Animal Care Commission of the College of Veterinary Medicine, Northwest A&F University. Every effort was made to minimize animal pain, suffering, and dis-tress. Two healthy horses, aged 2–3 years, were used for whole blood sample collection from Yangling Amusement Park (Shaanxi, China).

2.2. Isolation and characterization of horse Sp110 cDNA

Horse leukocytes were isolated from the blood sample by density-gradient centrifugation using Horse Leukocyte separating medium (HaoYang, China). Total RNA was isolated using the TRIzol® Reagent (Invitrogen, Life technologies) and first strand cDNA synthesis was done using the Reverse Transcription System (Takara). According to the computational analysis predicted horse SP110 mRNA sequence in the NCBI database, two pairs of primers, SP110-p1F\SP110-p1R and SP110-p2F\SP110-p2R (Table 1), were designed to clone the fragments of the cDNA. RACE ready cDNA was reverse-transcribed using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions. 3′ RACE primer SP110-3RCF (Table 1), was prepared based on the sequence of initially cloned partial fragment to determine the missing 3′ end of the ORF. And 5′ RACE primer, SP110-5RCR (Table 1), was designed to determine the transcription start site of horse SP110. Amplicons of the expected size were separated and cloned into p19T vector (Takara). The sequence information of the clones from independent PCR amplicons were determined by Applied Biosystems 3730XL Genetic Analyzer.

The full-length horse SP110 cDNA was assembled based on the initial cloned sequence contigs by ContigExpress software (Vector NTI Advance 11) and the open reading frames (ORFs) were identified by NCBI ORF Finder. Eventually, the novel horse SP110 variants and deduced amino acid sequences were determined, and Overlapping PCR was performed to construct the complete SP110 variant ORFs into pcDNA3.1 vectors.

2.3. Bioinformatic analysis

The gene structure of the horse SP110 variants, exon-intron nucleotide sequences, were determined by aligning the cloned cDNA sequences to horse genome sequence released in the NCBI GenBank. Through BLAST search analysis, we identified one contig (NCBI LOCUS NC_009149) that contained the region coding for the SP110 gene. The conserved domain analysis of the predicted SP110 amino acid sequence and the SP110 homologues of several animal species were carried out with Conserved-Domain Search of NCBI. Multiple sequence alignments of the nucleotide and protein sequences were carried out using CLUSTAL OMEGA of EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were constructed from the horse and other mammalian SP110 sequences using the Neighbor Joining method with 1000 bootstrap replications in the PHYLIP-3.69 program (http://evolution.genetics.washington.edu/phylip.html). SOPMA (http://npsa-prabi.ibcp.fr) and PHYRE [20] servers were used to predict the secondary structure and 3D homology modeling of the mature proteins.

2.4. Cell culture and transient transfections

RAW264.7 cells were grown in RPMI-1640 medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; GIBCO) at 37 °C in a humidified atmosphere with 5% CO_2. In order to compare the effects of horse SP110 variants on macrophages, we had to keep the expression of different recombinant proteins in the same level in RAW264.7. Macrophages were, separately, transfected with each SP110 variant expression cDNA3.1 vector in amount of 21 pmol plasmid per well on 12-well plates following the manual of FuGENE®HD Transfection Reagent (Promega, USA), and incubated for the indicated time.

2.5. Macrophage infection

MTB H37Ra, was obtained from the American Type Culture Collection (25177) and grown to log phase at 37 °C in Middlebrook 7H9 Medium (Fluka-SigmaAldrich, USA) with antibiotics (BBL™MGIT™PANTA™, BD, USA). In addition, mycobacteria was washed three times with sterile PBS to remove antibiotics before refrigerated storage, and then diluted to a certain concentration (~10^8 bacilli/mL) and stored at −80 °C as previous described [21]. RAW264.7 macrophages, after pretreated with transfection, were

Table 1
Sequence of oligonucleotide primers used for amplification of horse SP110.

Primer	Sequence (5′–3′)	Remarks
SP110-p1F	CTCAGTCTTCCAGGAGTGAT	Amplification of the SP110 fragment
SP110-p1R	TCACATTCCTTGGAGTTTTC	
SP110-p2F	GGAAGCCCATAGCAATCAGG	
SP110-p2R	TTTCGGTGGAGGGTGTTGAG	
SP110-3RCF	CATGGATCAGGCAACTCAAAGGATGG	Primers for 3′ RACE
SP110-5RCR	ATGAAGTGCTGGAGGAGAGCCTCTTC	Primers for 5′ RACE
SP110-OrfF	GCCATGGTCACCATG	Primers for cloning the transcript ORFs
SP110-Orf1R	TCATTTATGAAAAATA	
SP110-Orf2R	AATTCACCTGCTATTTA	

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