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Molecular characterization of the type I IFN receptor in two woodchuck species and detection of its expression in liver samples from woodchucks infected with woodchuck hepatitis virus (WHV)

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ABSTRACT

Type I interferons (IFN-α/β) serve as the first line of defense against viral infection and share the same type I IFN receptor (IFNAR) complex, which is composed of IFNAR1 and -2. The Eastern woodchuck (*Marmota monax*) and Chinese woodchuck (*Marmota himalayana*) are suitable for studying hepatitis B virus (HBV) infection. Here, the complete or partial sequences of the IFNARs of both species were obtained and analyzed. Small interference RNAs targeting wIFNAR1 and -2 specifically down-regulated the expression of wIFNAR1 and -2 and the IFN-stimulated gene MxA in a woodchuck cell line, respectively. IFNAR2 was significantly up-regulated in primary woodchuck hepatocytes stimulated with IFN-α or -γ. The expression of woodchuck IFNAR1 and -2 was decreased in woodchucks chronically infected with woodchuck hepatitis virus (WHV). These results are essential for studying type I IFN-related innate immunity and therapy in hepadnaviral infection in the woodchuck model.

1. Introduction

Type I interferons (IFNs), including IFN-α, -β, -ω, and -τ, serve as the first line of defense against viral infection by inducing the expression of numerous IFN-stimulated genes (ISGs) [1]. The activities of these cytokines are mediated by the type I IFN receptor (IFNAR) complex, which consists of IFNAR1 and -2 [1]. The interaction of type I IFNs with the IFNAR complex results in the activation of the JAK/STAT pathway [1–3]. This signaling pathway activates the transcription of numerous ISGs and ultimately leads to the biological functions of the type I IFNs.

Approximately 400 million individuals are chronically infected with hepatitis B virus (HBV) worldwide. Chronic HBV infection is one of the major causes of liver cirrhosis and hepatocellular carcinoma (HCC) [4,5]. Host genetic factors, including IFNAR1 and -2, may play an important role in determining the IFN response and the outcome of HBV infection. An IFNAR1 promoter variant was shown to significantly reduce IFNAR1 transcription levels by reducing its binding affinity towards high-mobility group protein B1 (HMGB1), which mediates the antiviral and immunoregulatory effects of IFN-α/β, and finally leads to a higher risk of chronic HBV infection [6,7]. A single-nucleotide polymorphism (SNP) in the IFNAR2 gene, IFNAR2 F8S, was also found to be associated with susceptibility to HBV infection in Africa and was shown to be important in determining the IFN response and clinical phenotypes of HBV infection in the Chinese Han population [8,9]. The expression of IFNAR-2 in monocytes may be used as a predictable parameter to evaluate the effect of IFN treatment in patients with chronic HBV infection [10]. Similarly, the expression level of the IFNARs was also found to have a predictive value in respond to IFN therapy in patients with chronic hepatitis C virus (HCV) infection [11–15]. Furthermore, co-infection with serologically silent HBV may

Abbreviations: IFN, interferon; ISG, IFN-stimulated genes; IFNAR, type I IFN receptor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HMGB1, high-mobility group protein B1; SNP, single-nucleotide polymorphism; HCV, hepatitis C virus; WHV, woodchuck hepatitis virus; PBMC, peripheral blood mononuclear cells; CDS, complete coding sequence; PWH, primary woodchuck hepatocytes; CRII, class II helical cytokine; IL15, interleukin 15.

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contribute to poor IFN response in patients with chronic HCV by down-regulating type I IFN receptor gene expression in the liver [16].

The infection of the Eastern woodchuck (*M. monax*) with woodchuck hepatitis virus (WHV) strongly resembles human HBV infection in its major virological, pathological, and immunological features. The WHV-infected woodchuck model is widely used to study HBV pathogenesis and evaluate antiviral drugs or therapeutic vaccines [17–23]. The Chinese woodchuck (*M. himalayana*) is phylogenetically closely related to the Eastern woodchuck (*M. monax*) [24]. We recently found that the Chinese woodchuck is susceptible to WHV infection [25]. Phylogenetic analysis indicates high homology in the IFN-related genes (IFN-α, MxA, IRF1, and STAT1) of these two woodchuck species [26,27]. This paper identified the type I IFN receptors (including IFNAR1 and -2) of *M. himalayana* and *M. monax* and measured their bioactivities in the IFN signaling pathway by using siRNA interference in the WH12/6 woodchuck cell line. We also analyzed the IFNAR1 and -2 expression in woodchuck primary hepatocytes pulsed with IFN and WHV-infected woodchuck livers. Our data provide basic information regarding the use of the woodchuck model in the study of type I IFN-related innate immunity and therapy in HBV infection.

2. Results

2.1. Cloning and sequence analysis of M. himalayana IFNAR1 and -2

Total RNA was purified from the peripheral blood mononuclear cells (PBMCs) of *M. himalayana*, and the cDNA sequences of *M. himalayana* IFNAR1 and -2 (shortened as mhIFNAR1 and -2, respectively) was obtained by RT-PCR. The specific PCR product was generated using the conserved IFNAR primer pairs and cloned into pMDT-18. Four clones of each gene were selected for sequencing. The complete coding sequence (CDS) of mhIFNAR1 (1662 base pairs (bp)) was obtained, the predicted protein includes 553 amino acid (aa) residues. The sequence information was submitted to Genbank (Accession Number JN379357). The sequence of mhIFNAR1 shows high homology with the IFNAR1 of other species, ranging from 60% to 80% at the nucleotide (nt) level and 48% to 67% at the aa level (Table 1). The alignment analysis indicated that eight cysteine residues (Cys79, Cys87, Cys199, Cys220, Cys283, Cys291, Cys399, and Cys422) in the extracellular domain and four tyrosine residues (Tyr462, Tyr477, Tyr523, and Tyr534) in the intracellular domain are conserved. An InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) analysis indicated that the mhIFNAR1 precursor has a putative signal peptide 16 aa in length and that the mature protein contains 537 aa. The predicted protein has a short transmembrane region (433–453 aa). Two fibronectin type III domains (30–136 and 229–340 aa) and two interfer-bind domains (126–224 and 332–426 aa) exist in the extracellular domain of the predicted mhIFNAR1 protein (Fig. 1A).

The partial CDS of mhIFNAR2 (consisting of 1184 bp) was identified, and the deduced aa sequence has 394 aa residues (Genbank accession number JN379359). The mhIFNAR2 sequence shows high homology with the IFNAR2 of other species, ranging from 65% to 72% at the nt level and 49% to 59% at the aa level (Table 1). The alignment analysis indicated that four cysteine residues (Cys35, Cys43, Cys160, and Cys180) in the extracellular domain and four tyrosine residues (Tyr222, Tyr269, Tyr271, and Tyr290) in the intracellular domain are conserved. The InterProScan analysis indicated that the predicted mhIFNAR2 aa sequence has a short transmembrane region (197–217 aa), one fibronectin type III domain (1–85 aa) and one interfer-bind domain (84–181 aa) in the intracellular domain (Fig. 1B).

Table 1
Homology of mhIFNAR1 and -2 with the counterparts of *M. monax* and the other mammalian species.

Species	mhIFNAR1		mhIFNAR2	
	nt.	aa.	nt.	aa.
M. monax	99.33	98.99	98.05	97.06
Human	80.14	67.09	72.91	59.49
Pig	74.85	61.48	69.37	51.90
Cow	77.32	63.11	69.37	52.15
Mouse	65.46	48.82	65.15	49.62
Rat	60.55	49.19	65.96	50.25

Note: The accession number of IFNAR1 sequences used for alignment: JN379357 (*M. himalayana*), JN379359 (*M. monax*), NM_000629 (human), NM_174552 (cow), NM_213772 (pig), NM_010508 (mouse), NM_001105893 (rat).
The accession number of IFNAR2 sequences used for alignment: JN379358 (*M. himalayana*), JN379360 (*M. monax*), NM_207585 (human), NM_174553 (cow), NM_001204775 (pig), NM_010509 (mouse), NM_001204775 (rat).

2.2. Cloning and sequence analysis of M. monax IFNAR1 and -2

We also identified the partial CDS of *M. monax* IFNAR1 (396–1284 nt) and -2 (799–1122 nt) (shortened as wIFNAR1 and -2, respectively). The sequences were submitted to Genbank with accession numbers of JN379359 (wIFNAR1) and JN379360 (wIFNAR2). The predicted wIFNAR1 and -2 proteins have 296 and 107 aa residues, respectively. Both wIFNARs have only six nt substitutions (Supplementary Fig. 1) and three aa substitutions (Supplementary Fig. 2) and show high homology to their *M. himalayana* counterparts (Table 1). The homology between wIFNAR1 and mhIFNAR1 is 99.33% at the nt level and 98.99% at the aa level, and the homology of wIFNAR2 and mhIFNAR2 is 98.05% at the nt level and 97.06% at the aa level (Table 1). A phylogenetic analysis indicated that mhIFNAR and wIFNAR are closely related (Fig. 2).

2.3. Knockdown of IFNARs by siRNA abolishes MxA expression after IFN stimulation in the W12/6 woodchuck fibroblastoma cell line

To clarify the bioactivity of the wIFNARs and mhIFNARs obtained above, we analyzed the effect of siRNA targeting woodchuck IFNAR (siIFNAR1 and -2) on the expression of ISG (MxA). WH12/6 woodchuck fibroblastoma cells were first transfected with siIFNAR1 and -2, respectively, and then stimulated with wIFN-α. The wIFNAR1, wIFNAR2, and MxA mRNA levels were then measured by real-time RT-PCR and normalized to β-actin. IFNAR1, IFNAR2, and MxA were only expressed at the baseline level in the unstimulated W12/6 cells and were only slightly reduced in the siIFNAR1 and siIFNAR2 transfected cells (Fig. 3A and B). After stimulation with wIFN-α, the expression of IFNAR1 and MxA mRNA increased dramatically, and the siIFNAR1-mediated reduction in IFNAR1 and MxA mRNA levels became more pronounced in the W12/6 cells (for IFNAR1, siIFNAR1 vs. siIRR: 4072.79 ± 360.69 vs. 19348.65 ± 6917.32, $P = 0.019$; for MxA, siIFNAR2 vs. siIRR: 8.04 ± 3.04 vs. 136.54 ± 9.51, $P = 0.001$) (Fig. 3C). Similar results were obtained in the cells transfected with siIFNAR2 (for IFNAR2, siIFNAR2 vs. siIRR: 1438.18 ± 300.57 vs. 42052.56 ± 21594.84, $P = 0.031$; for MxA, siIFNAR2 vs. siIRR: 16.22 ± 11.78 vs. 128.99 ± 25.52, $P = 0.002$) (Fig. 3D). These data suggest that both the wIFNAR1 and -2 specific siRNAs could effectively knock down the expression of IFNAR and downstream MxA expression in the woodchuck cell line after stimulation with IFN-α.

2.4. Both IFN-α and -γ up-regulated the expression of IFNAR2 in primary woodchuck hepatocytes (PWHs)

We also measured the IFNAR expression in PWHs from naïve woodchucks (*M. monax*) with or without IFN stimulation. PWHs