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Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells

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***Background/Aims*: We previously established hepatitis C virus (HCV) replicon-harboring cell lines possessing two interferon (IFN)-resistant phenotypes: a partially resistant phenotype (αR series) and a severely resistant phenotype (βR series). We recently found that the severe IFN resistance of the βR-series cells is caused by the functional disruption of type I IFN receptors. Here, we aimed to clarify the mechanism(s) underlying the partial IFN resistance of the αR-series cells.**

***Methods*: αR-series cells were pre-treated with 5-azacytidine to evaluate the effects of DNA demethylation on IFN resistance. cDNA microarray analysis was carried out in order to compare 1αR cells, which belong to the αR series, treated with both 5-azacytidine and IFN-α with cells treated with 5-azacytidine or IFN-α alone.**

***Results*: We found that the IFN-resistant phenotype of αR-series cells was impaired by treatment with 5-azacytidine. cDNA microarray analysis identified seven IFN-stimulated genes, which were up-regulated by 5-azacytidine treatment. We demonstrated here that the ectopic expression of each of these seven genes in 1αR cells frequently weakened the IFN resistance of these cells.**

***Conclusions*: The present results suggest that the epigenetic silencing of IFN-stimulated genes is implicated in the acquisition of a partially IFN-resistant phenotype of HCV replicon-harboring cells.**

***Keywords*: IFN resistance; HCV replicon; DNA methylation; cDNA microarray; Epigenetic silencing**

1. Introduction

Persistent infection by the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which can progress to liver cirrhosis and hepatocellular carcinoma [3]. HCV is an enveloped RNA virus belonging to the family Flaviviridae, the genome of which consists of a positive-stranded 9.6-kb RNA encoding at least 10 structural and non-structural proteins [4]. Since, at least 170 million people are currently infected with HCV worldwide, this type of infection constitutes a global health problem [5]. Interferon (IFN)-α/ribavirin combination therapy is currently the standard clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to approximately half of these patients [6]. This clinical finding suggests that HCV is resistant to the antiviral effects of IFN, and that HCV proteins directly or indirectly attenuate those effects [7].

Although HCV replicon system harboring autonomously replicating HCV subgenomic RNA containing the non-structural region [8] provides a powerful tool for various HCV studies, all of the HCV replicons established to date

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Abbreviations: aa, amino acid; 5-azaC, 5-azacytidine; CH, chronic hepatitis; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN receptor; IRF-1, IFN regulatory factor 1; ISG, IFN-stimulated gene; RT-PCR, reverse transcription-polymerase chain reaction.

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have been highly sensitive to IFN [9–12]. Based on our assumption that prolonged IFN treatment might change HCV replicons from the IFN-sensitive phenotype into an IFN-resistant phenotype, we established nine HCV replicon-harboring cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series; 1αR, 3αR, 4αR, 5αR, and αRmix) and a severely resistant phenotype (βR series; 1βR, 3βR, 4βR, and 5βR) obtained by IFN-α and IFN-β treatment, respectively [13]. αR- and βR-series cells were derived from clones 1, 3, 4, and 5, and 1αR, 3αR, 4αR, and 5αR cells were counterparts of 1βR, 3βR, 4βR, and 5βR cells, respectively [13]. Although genetic analysis of these replicons identified one common amino acid (aa) substitution and several cell line-specific aa substitutions, we failed to obtain the evidence of the direct involvement of these aa substitutions to IFN resistance [14]. However, we found frequent non-sense mutations and deletions in type I IFN receptor (IFNAR) genes (IFNAR1 and IFNAR2c) in the βR-series cells, but such mutations were rarely observed in the αR-series cells [14]. Since we demonstrated that the ectopic expression of wild-type IFNAR in the βR-series cells restored IFN signaling, we determined that the functional disruption of type I IFNAR was responsible for this type of resistance [14]. However, the mechanism underlying the partial IFN-resistance of αR-series cells remains unclear. Since, the expression levels of IFNARs, Tyk2, and Jak1 were not lower in the αR-series cells [13,14], the functional degeneration of other cellular factor(s) involved in IFN signaling or IFN-stimulated gene(s) (ISG) was thought to contribute to the acquisition of IFN resistance.

As one potential mechanism for the partial resistance, we assumed that the epigenetic silencing of some ISGs, which are known to be involved in the anti-HCV activity of IFN, by DNA methylation around the promoter region might convert HCV replicon-harboring cells from the IFN-sensitive phenotype to the IFN-resistant phenotype. To evaluate our hypothesis, we examined whether or not pre-treatment of αR-series cells with 5-azacytidine (5-azaC), an inhibitor of DNA methyltransferase and an inducer of gene suppressed by DNA methylation, could alter the IFN sensitivity of the cells.

Here, we report that the epigenetic silencing of ISGs is implicated in the IFN-resistance of αR-series cells. We have also identified several ISGs that are up-regulated by 5-azaC treatment and weaken the IFN resistance of αR-series cells.

2. Materials and methods

2.1. Cell cultures

HCV replicon-harboring cells and cured 6Mc cells [14], from which the HCV replicons had been eliminated by IFN-γ treatment, were maintained as described previously [13].

2.2. Analysis of IFN sensitivity

HCV replicon-harboring cells were treated with 5-azaC (2–10 μM) (A-2385, Sigma, St Louis, MO) for 2 weeks. Then, human IFN-α (I-2396; Sigma) was added to the cells (with or without pre-treatment with 5-azaC) as described previously [12,13]. After 3 weeks in culture, the colonies obtained on the culture dishes were stained with Coomassie brilliant blue (CBB) as described previously [15].

2.3. Quantitative analysis of HCV replicon RNA

The quantification of HCV RNA was carried out to monitor the antiviral effects of IFN-α, and was performed by real-time LightCycler polymerase chain reaction (PCR) as described previously [16,17].

2.4. Construction of replicon plasmid and RNA synthesis

The non-structural region (6.1 kb) fragment of a 1βR1 clone obtained from 1βR cells [13] was digested with SpeI and BsiWI, and the digested

Table 1
The primers used for RT-PCR analysis of mRNA expression

Gene	Direction	Nucleotide sequence	Products (bp)	Cycles
IFI27	Forward	gttttgcccctggccaggattgct	252	27
	Reverse	aatggagcccaggatgaacttggt		
9-27	Forward	tcttcttgaactggtgctgtctggg	191	28
	Reverse	agagccgaataccagtgacaggat		
LMP2	Forward	atggaaccctgggaggaatgctg	145	27
	Reverse	gcaatagcgtctgtggtgaagcg		
LMP7	Forward	ctgggataagaagggtcctggac	293	27
	Reverse	tactggtgcagcaggtcactggac		
Viperin	Forward	tggagcgccacaaagaagtgtcct	240	27
	Reverse	ccagcttcagatcagccttactcc		
IFI44	Forward	tgtggctttgctcactcatgtgga	227	31
	Reverse	cagcccatagcattcgtctcagag		
IFIT2	Forward	aggccatccaccactttatagagg	272	28
	Reverse	tgggcaccacatctctattctcca		
ISG56	Forward	tagccaacatgtcctcacagac	396	32
	Reverse	tcttctaccactggtttcatgc		
GAPDH	Forward	gactcatgaccacagtccatgc	334	26
	Reverse	gaggagaccacctggtgctcag		