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RESEARCH ARTICLE

MicroRNA-548 down-regulates host antiviral response via direct targeting of IFN-λ1

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ABSTRACT

Interferon (IFN)-mediated pathways are a crucial part of the cellular response against viral infection. Type III IFNs, which include IFN-λ1, 2 and 3, mediate antiviral responses similar to Type I IFNs via a distinct receptor complex. IFN-λ1 is more effective than the other two members. Transcription of IFN-λ1 requires activation of IRF3/7 and nuclear factor-kappa B (NF-κB), similar to the transcriptional mechanism of Type I IFNs. Using reporter assays, we discovered that viral infection induced both IFN-λ1 promoter activity and that of the 3′-untranslated region (UTR), indicating that IFN-λ1 expression is also regulated at the post-transcriptional level. After analysis with microRNA (miRNA) prediction programs and 3′UTR targeting site assays, the miRNA-548 family, including miR-548b-5p, miR-548c-5p, miR-548i, miR-548j, and miR-548n, was identified to target the 3′UTR of IFN-λ1. Further study demonstrated that miRNA-548 mimics down-regulated the expression of IFN-λ1. In contrast, their inhibitors, the complementary RNAs, enhanced the expression of IFN-λ1 and IFN-stimulated genes. Furthermore, miRNA-548 mimics promoted infection by enterovirus-71 (EV71) and vesicular stomatitis virus (VSV), whereas their inhibitors significantly suppressed the replication of EV71 and VSV. Endogenous miRNA-548 levels were suppressed during viral infection. In conclusion, our results suggest that miRNA-548 regulates host antiviral response via direct targeting of IFN-λ1, which may offer a potential candidate for antiviral therapy.

KEYWORDS microRNA-548, interferon-λ1, viral infection, antiviral response

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules of 18-24 nucleotides. These RNAs play important roles in the control of gene expression to regulate various biological processes, such as development, cell differentiation, cell proliferation, and apoptosis (Bartel, 2004). The miRNAs target the 3′-untranslated region (UTR) of messenger RNA (mRNA) and regulate the expression of numerous genes by catalyzing mRNA cleavage (Hutvagner and Zamore, 2002) or repressing mRNA translation (Doench and Sharp, 2004). Hsa-miR-548 is a recently discovered human miRNA gene family that is derived from miniature inverted-repeat transposable elements (MITEs). Consistent with their origin from MITEs, miR-548 genes are primate-specific and have many potential paralogs in the human genome. Thousands of putative miR-548 target genes have been identified, and analysis of their expression profiles and functional affinities suggests cancer-related regulatory roles for miR-548 (Piriyapongsa and Jordan, 2007).

The interferon (IFN) family represents key components of the innate immune response and is the first line of defense against virus infection. Three classes of IFN have been identified, and the type I, II, and III IFNs are classified according to the receptor complex used for signaling (Muller et al., 1994; Kotenko et al., 2003). Type II IFN consists of the single IFN-γ gene and mediates broad immune responses to different pathogens (Farber, 1990). Type I IFNs, which comprise 13 IFN-α members and a single IFN-β, IFN-κ, IFN-ε, and IFN-ω in humans, engage the ubiquitously expressed IFN-α receptor (IFNAR) complex, which is composed of IFNAR1 and IFNAR2 (Pestka, 1997; LaFleur et al., 2001; Conklin et al., 2002). The well-characterized type I IFNs are essential for mounting a robust host response against viral infection. Type III IFNs are a newly designated IFN family that consists of

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IFN-λ1, IFN-λ2, and IFN-λ3 (also designated IL-29, IL-28A, and IL-28B, respectively). Type III IFNs act via a distinct receptor complex composed of one exclusive chain and a second chain that is shared with the receptor for interleukin(IL)-10, IL-22, and IL-26 (all belong to the family of IL-10-related cytokines) (Kotenko et al., 2003). In spite of different receptors, type III IFNs induce reactions identical to those induced by type I IFNs. These reactions result in activation of the Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways, subsequent activation of the transcriptional factors, gamma activated sequence (GAS) and IFN stimulated response element (ISRE), and consequent induction of interferon stimulated genes (ISGs) expression (Maher et al., 2008; Bandi et al., 2010). Type III IFNs interfere with the multiplication of several human and murine viruses, and among the three members of this type, IFN-λ1 exerts the best antiviral effect (Sheppard et al., 2003; Robek et al., 2005; Hong et al., 2007; Almeida et al., 2008).

In addition to the similarities in antiviral functions, the regulation of expression of the type III IFNs is similar to that of the type I IFNs. Expression of both types is regulated by viral infection and requires activation of the transcription factors NF-κB and interferon regulatory factor (IRF)-3 and IRF7. The IFN-λ1 gene is regulated by virus-activated IRF3 and IRF7 and resembles the IFN-β gene. The IFN-λ2/3 genes, which are expressed at significantly lower levels, are controlled mainly by IRF7, which is similar to the IFN-α genes (Osterlund et al., 2007).

Although the IFNs are induced by viruses, some viral components inhibit the IFN signaling pathway to improve viral survival. Hepatitis B virus X protein suppresses virus-triggered IRF3 activation and IFN induction by disrupting the virus-induced signaling adapter (VISA)-associated complex (Wang et al., 2010). EV71 3C protein cleaves the Toll-like receptor 3 (TLR3) adaptor protein (Lei et al., 2011) and inhibits the retinoid acid-inducible gene I (RIG-I)- mediated IRF3 activation (Lei et al., 2010), which inhibits IFN-mediated antiviral response. In a hepatoma line, HCV infection induced transient IRF3 activation, but the activation was not sustained (Loo et al., 2006). The HCV NS3/4A exerts the same functions as the EV71 3C protein (Li et al., 2005; Milliken et al., 2005). These findings suggest that IFN expression during viral infection is very low; however, ISGs are highly expressed in liver biopsies during HCV infection, even in tissues lacking detectable IFN-α/β expression (Bigger et al., 2001; Sarasin-Filipowicz et al., 2008). Recent reports indicated that HCV induces expression of IFN-λ1 and ISGs and reduced expression of Type I IFNs in primary liver cells (Marukian et al., 2011) and in chimpanzees (Park et al., 2012). In this study, we investigated IFN-λ1 expression at the translational level and found that miR-548 specifically targets the 3′UTR of IFN-λ1 and regulates the expression of IFN-λ1. Our results provide a novel mechanism for the post-transcriptional regulation of IFN-λ1 during viral infection.

RESULTS

IFN-λ1 3′UTR activity is responsive to viral infection

We utilized several virus infection models to investigate the induction of IFN-λ1 expression during viral infection. First, the HBV-expressing plasmid pHBV-1.3 was transfected into HepG2 cells to mimic infection with the HBV virus. In addition, human embryonal rhabdomyosarcoma (RD) and HepG2 cells (a hepatoma cell line) were infected with EV71 (multiplicity of infection, MOI = 5) and VSV (MOI = 5), respectively. The mRNA levels of IFN- λ1 were analyzed in infected cells and controls. IFN-λ1 mRNA levels were induced approximately 8-fold, 30-fold, and 80-fold in the presence of HBV, EV71, and VSV, respectively (Fig. 1A). Then the activity of a 1903-bp IFN-λ1 promoter reporter was tested in these models. The promoter activities were stimulated to a lesser degree than the cognate mRNA levels induced by HBV, EV71, and VSV. In particular, only a 1.8-fold increase in IFN- λ1 promoter activity was detected during EV71 infection (Fig. 1B). The stability or translational capability of IFN-λ1 mRNA also contributes to the expression of this cytokine. The 3′UTR plays a pivotal role in the regulation of gene expression at the post-transcriptional level. Therefore, we performed IFN-λ1 3′UTR luciferase reporter assays to determine the effects of viral infection on 3′UTR activity (Yue et al., 2012). Upon transfection of the reporter plasmid, significant induction of the 3′UTR activity was observed in the presence of HBV, EV71 and VSV (Fig. 1C). These results indicate that expression of IFN-λ1 is regulated at both the transcriptional and the post-transcriptional levels and that the 3′UTR is involved in virus-induced expression of this gene.

MiR-548 targets the 3′UTR of IFN-λ1 and alters its activity

MiRNAs play important roles in various biological processes, including viral infection and the related pathological effects. Bioinformatics analyses suggested that miRNAs regulate the expression of more than 30% of all human genes. These RNAs target the 3′UTR of mRNAs and regulate the expression of numerous genes by affecting the cleavage of mRNA or translation. The IFN-λ1 3′UTR was analyzed with programs for miRNA targeting prediction, including PicTar, TargetScan, miRanda, and miRGen. Several high-scoring candidate miRNAs (miR-548b-5p, miR-548c-5p, miR-548i, miR-548j, and miR-548n), which belong to the miR-548 family and share consensus sequences, were selected (Fig. 2A). To examine whether these miRNAs affect the function of IFN-λ1 3′UTR, synthetic double-stranded miRNA mimics and their inhibitors, single-stranded complementary RNAs for the inhibition of endogenous miRNAs, were used to perform IFN-λ1 3′UTR activity assays. Indeed, miR-548 mimics inhibited the 3′UTR activity, and in contrast, their inhibitors

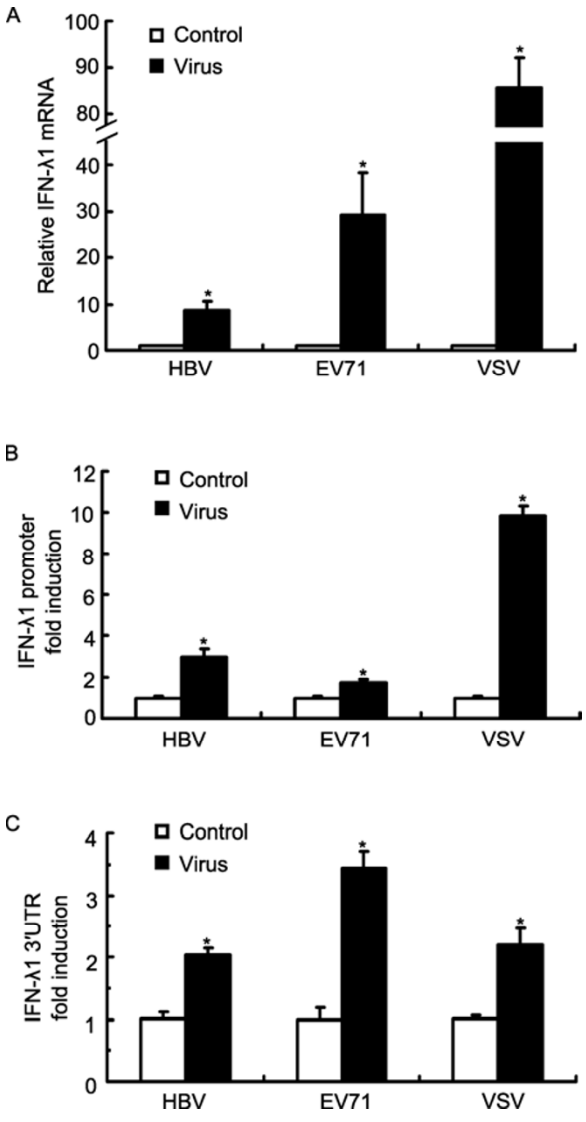

Figure 1. Involvement of the 3′UTR in viral regulation of INF-λ1 expression. HepG2 cells were transfected with pHBV-1.3 or empty vector for 48 h. RD cells were infected with EV71 (MOI = 5) for 12 h, and HepG2 cells were infected with VSV (MOI = 5) for 12 h. Then, the cells in each group were harvested for detection. (A) IFN-λ1 mRNA levels were analyzed by real-time PCR. (B) The IFN-λ1 promoter (1903 bp) reporter plasmid was co-transfected with pHBV-1.3 or empty vector into HepG2 cells. The plasmid was also transfected into RD or HepG2 cells 24 h before EV71 or VSV infection, respectively. The harvested cells were used for luciferase activity analysis. (C) The IFN-λ1 3′UTR reporter plasmid was examined as for the experiment described in B. *$P < 0.05$

controls. To verify the predicted targeting site on the UTR, a mutant construct was generated from the IFN-λ1 3′UTR reporter plasmid. The mutant was then co-transfected along with the synthetic mature miRNA mimics or inhibitors to test the dependence of the activity on the targeting site. This experiment revealed that the activity of the mutant 3′UTR was not altered by miR-548 mimics or the inhibitors (Fig. 2B). These results demonstrated that miR-548 regulates the activity of the IFN-λ1 3′UTR by directly targeting the sequence at the predicted site.

MiR-548 regulates endogenous IFN-λ1 expression

Since miR-548 targeted the IFN-λ1 3′UTR, we predicted that endogenous IFN-λ1 expression was also affected by miR-548 mimics and their inhibitors. To test this hypothesis, HepG2 cells were transfected with miR-548 mimics followed by infection with VSV. A specific shRNA for IFN-λ1 was used as positive control (Yu et al., 2011). Both virus-induced IFN-λ1 mRNA and protein were significantly inhibited by miR-548 (Fig. 3A). The same inhibition was observed for the expression of ISGs, the human myxovirus resistance protein 1 (MxA) and 2′,5′-oligoadenylate synthetase-1 (OAS1) (Fig. 3B). Then IFN-λ1 expression was detected in HepG2 cells transfected with miR-548 inhibitors or nonsense RNA controls, and poly I:C was used as positive control (Siren et al., 2005). The result showed that the inhibitors of miR-548b-5p, miR-548c-5p, miR-548i, and miR-548j increased the IFN-λ1 levels (Fig. 3C). These results showed that the endogenous IFN-λ1 and ISGs were regulated by miR-548. These findings imply that miR-548 and the inhibitors regulate host antiviral response.

We performed a test of mRNA decay to determine whether miR-548 regulated IFN-λ1 expression through translational repression or mRNA degradation mechanism. HepG2 cells were treated with poly I:C to stimulate IFN-λ1 expression and transfected with miR-548i or specific shRNA for IFN-λ1, followed by actinomycin-D treatment for transcriptional suppression. Both the total and relative secretion of IFN-λ1 protein within the indicated time intervals decreased gradually. The shRNA and miR-548 further inhibited the protein production, comparing with their negative controls (Fig. 3D). Remarkably, miR-548 induced a quicker decay of IFN-λ1 mRNA similar to the shRNA (Fig. 3E). This indicates that miR-548i may share the same mechanism of interfering RNA, which induces IFN-λ1 mRNA degradation. The analysis of IFN-λ1 protein/mRNA ratio showed that miR-548 has no apparent translational repressing activity (Fig. 3F), demonstrating that miR-548 inhibited IFN-λ1 expression mainly through mRNA degradation mechanism.

Viral replication is affected by miR-548 and the inhibitors

We next examined whether these inhibitors inhibited viral infection or replication. Antiviral assays in the VSV infection model generated promising results. Following infection of cells with VSV-eGFP, which is a recombinant virus that expresses green fluorescent protein, the GFP expression level represents viral replication as previously described (Gack et al., 2007). Both the microphotographs and flow

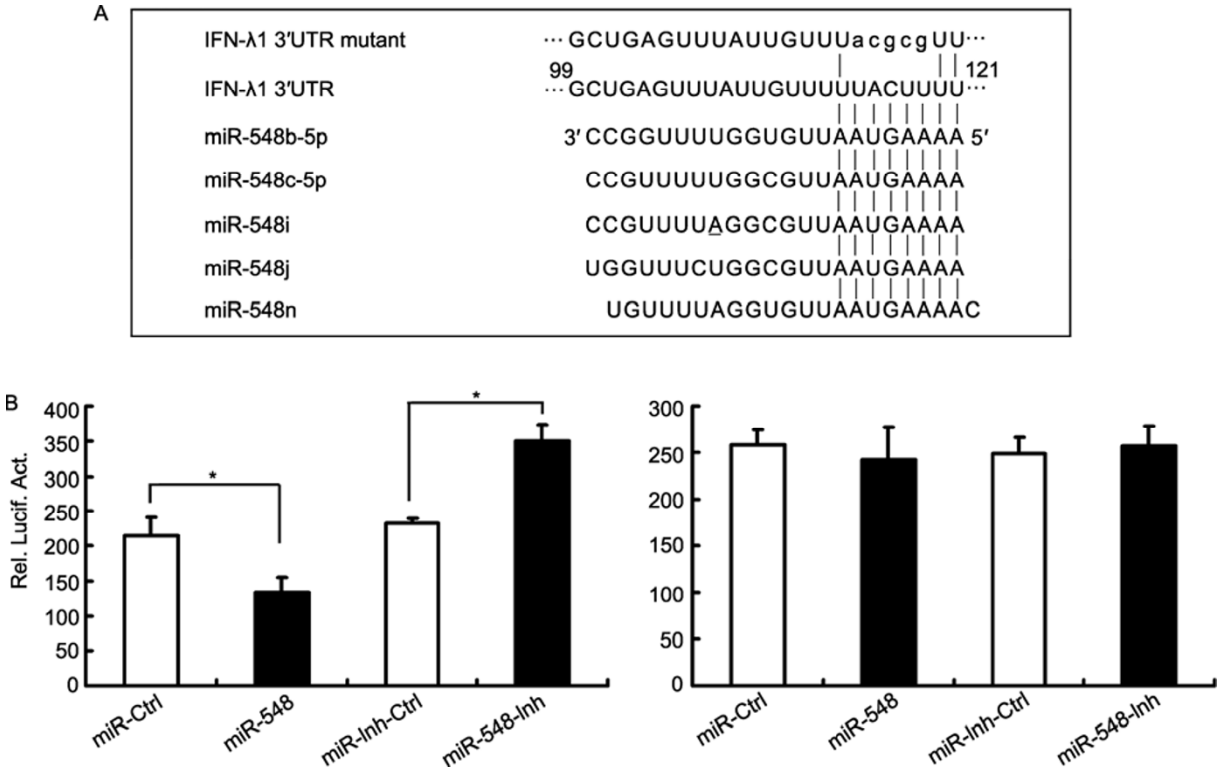

Figure 2. Analysis of IFN-λ1 3′UTR and targeting of miR-548. (A) The predicted miR-548 targeting site in the IFN-λ1 3′UTR and the UTR mutant. (B) IFN-λ1 3′UTR luciferase reporter plasmid was co-transfected into HepG2 cells with 100 nmol/L of miR-548 mimics and inhibitors (20 nmol/L each subtype of the five miRNAs displayed in A) or 100 nmol/L nonsense control miRNAs. After 48 h, the luciferase activities were analyzed (left panel). The same experiment was performed with the mutant IFN-λ1 3′UTR luciferase reporter plasmid (right panel).*$P < 0.05$

cytometric data showed that miR-548 inhibitors effectively exhibited the strongest antiviral activity, and the activity of this inhibitor was equivalent to that of recombination human IFN-α and IFN-λ1 (Fig. 4). These antiviral assays were also performed with EV71, and the results showed EV71 VP1 levels in RD cells transfected with miR-548 inhibitors were considerably lower than the nonsense inhibitor control (Fig. 5A). The viral RNA produced in this experiment was also measured. The viral RNA copies were reduced by approximately 2 logs by the miR-548i inhibitor (Fig. 5B). As expected, transfection of miR-548 mimics increased EV71 VP1 expression significantly (Fig. 5C). To further verify the antiviral action of miR-548 inhibitor through specific regulation of IFN-λ1, IFN-λ1 silence by specific shRNA was performed in the antiviral assay. The result showed that knocking-down of IFN-λ1 almost abolished the antiviral effect of miR-548 inhibitor (Fig. 5D). These data prove that miR-548 inhibitors suppressed viral replication through regulation of INF-λ1.

Previous reports indicated that this primate-specific miRNA family may play regulatory roles in early differentiation, growth regulation, and cancer development (Piriyapongsa and Jordan, 2007; Lin et al., 2010). To probe whether the effects of miR548 mimics and inhibitors have an effect on the growth or generation cycles of cells, MTT assays were performed, and the cell viabilities were evaluated. Transfection with any of the miR-548 mimics and inhibitors did not affect cell growth compared to the effects following treatment with the Lipofectamine 2000 reagent control (Fig. 5E and 5F). Together, our results provide evidence that miR-548 mimics and inhibitors modulate viral replication via regulation of IFN-λ1 expression in the absence of any effects on cell growth.

Endogenous miR-548 level is suppressed during viral infection

Our studies indicated that miR-548 inhibited IFN-λ1 expression via targeting the 3′UTR of the mRNA and that the activity of IFN-λ1 3′UTR is upregulated by virus infection. These data indicate that endogenous miR-548 expression is likely to be suppressed during viral infection. To test this presumption, we transfected HepG2 cells with the HBV-expression plasmid pHBV-1.3 or empty vector and examined the endogenous miR-548 levels. As expected, an obvious decrease of this miRNA was observed in the presence of HBV (Fig. 6A). To explore miR-548 expression in EV71 and VSV infection

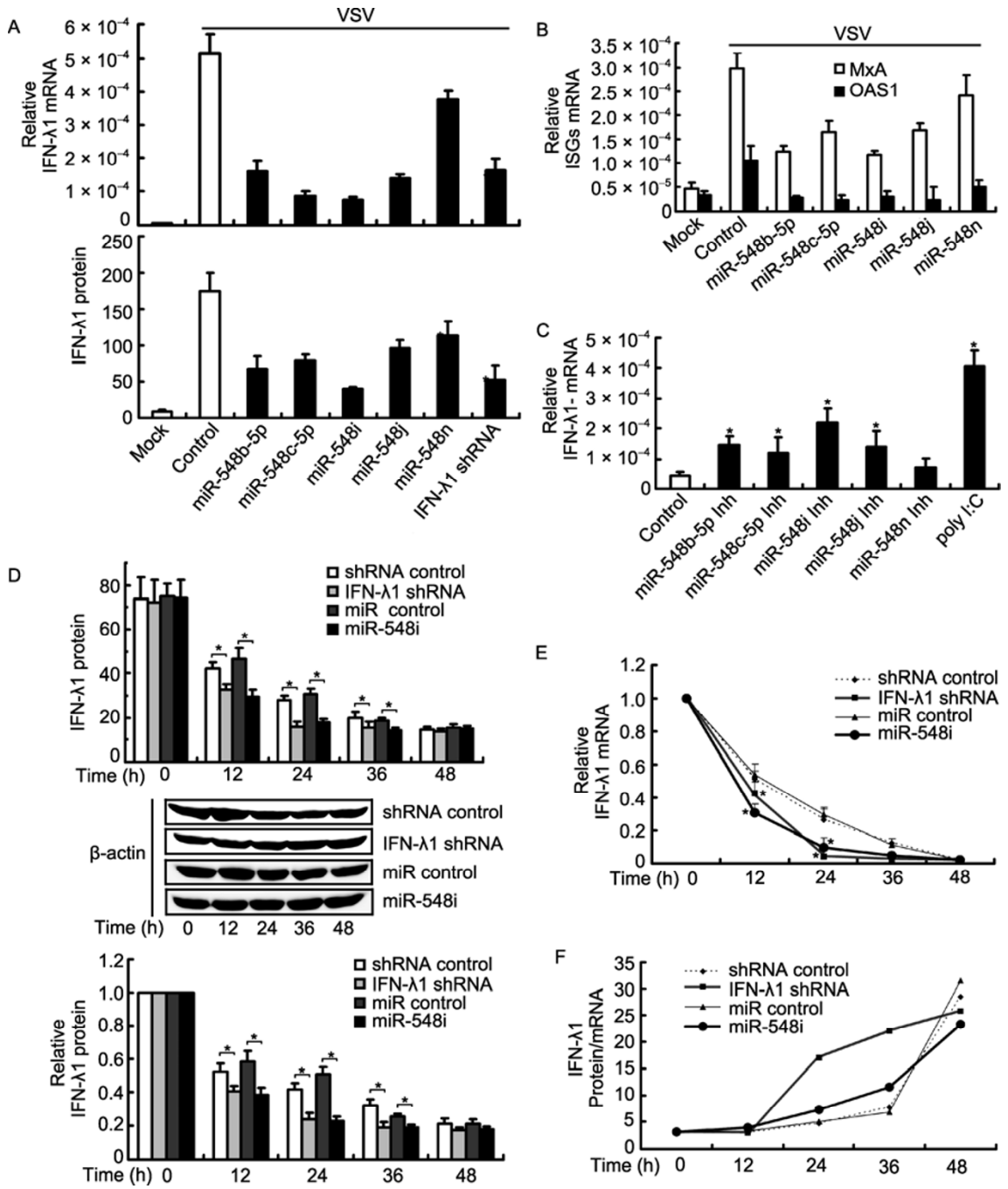

Figure 3. Effect of miR-548 on IFN-λ1 expression. (A and B) HepG2 cells were transfected with the five subtypes of miR-548 mimics, IFN-λ1 specific shRNA or controls. Twenty-four hours later, the cells were infected with VSV (MOI = 5) for 8 h. Then the cell culture supernatants were used for IFN-λ1 detection by ELISA (A, top panel). The relative mRNA levels of IFN-λ1 (A, bottom panel) and ISGs (B) in cell lysates were analyzed by real-time PCR. Samples transfected with controls and then infected with VSV were used as control, and HepG2 cells transfected with controls were used as mock samples. (C) HepG2 cells were transfected with miR-548 inhibitors or control RNA for 24 h, relative mRNA levels of endogenous IFN-λ1 were analyzed by real-time PCR. Poly I:C (50 μg/mL) was used as a positive control. (D) Poly I:C-treated HepG2 cells were transfected with miR-548i, IFN-λ1 specific shRNA or controls, followed by treatment with actinomycin-D (5 μg/mL). Secreted IFN-λ1 protein in the supernatants were collected at 0, 12, 24, 36 and 48 h and measured with ELISA kit (top panel). Cells were harvested for β-actin measurement by western blot (middle panel). The bands of β-actin were quantified with densitometric image analysis software. IFN-λ1 protein levels were normalized by band densities of β-actin (bottom panel). (E) Relative IFN-λ1 mRNA levels in samples harvested in D were analyzed by real-time PCR using GAPDH as an internal control. (F) IFN-λ1 protein/mRNA ratio was calculated for all samples in D and E. * $P < 0.05$

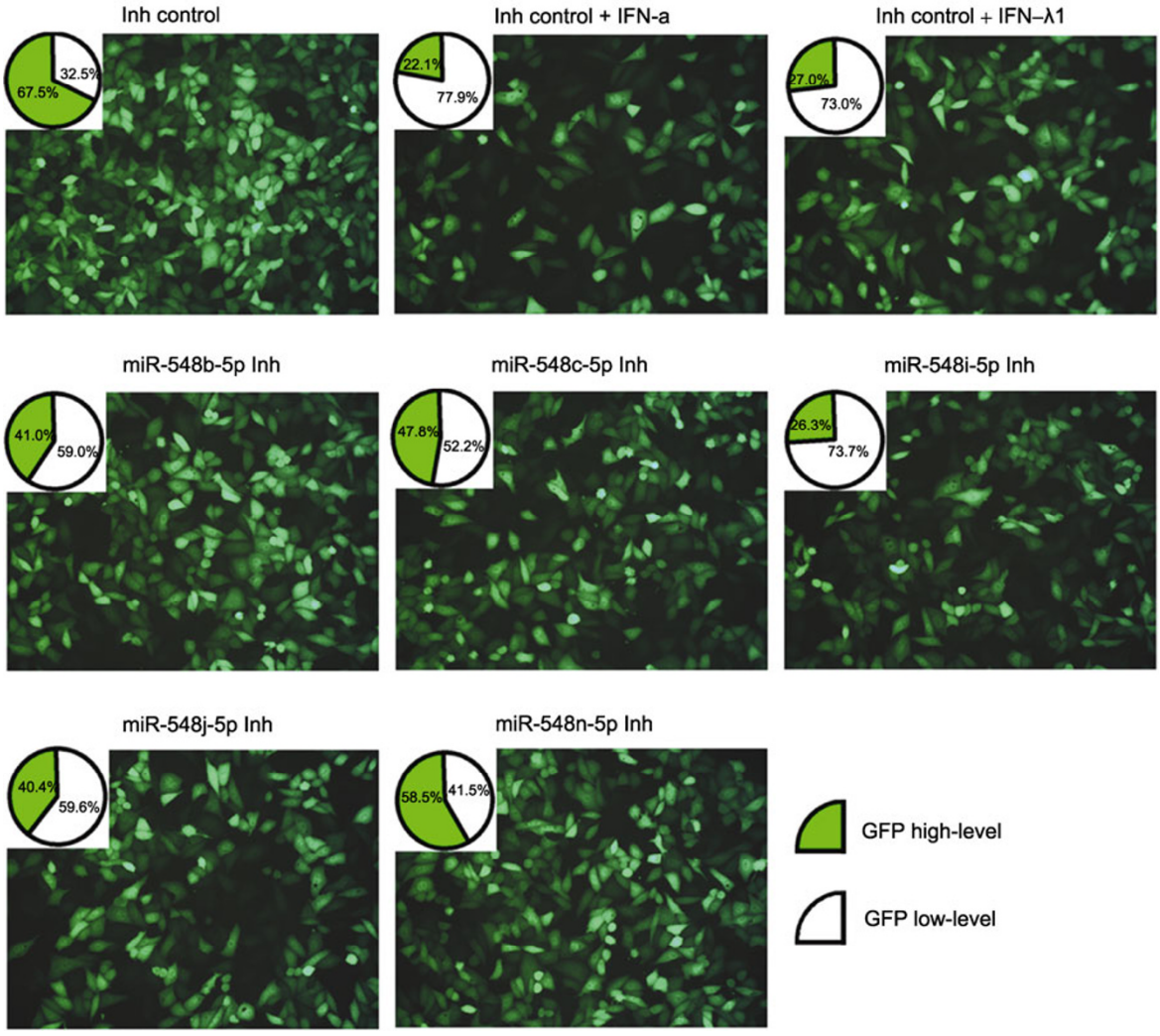

Figure 4. Antiviral assay following VSV infection. HepG2 cells were transfected with the inhibitors of the five subtypes of miR-548 or nonsense miRNA inhibitor as a control. After 24 h, the cells were infected with VSV-eGFP (MOI = 5) for 12 h. Two samples that were transfected with control miRNA inhibitor were treated with IFN-α (100 IU/mL) or IFN-λ1 (100 ng/mL) for 12 h before infection. Microphotographs were taken, and the cells were subjected to flow cytometry analysis for GFP expression (data shown in insets).

systems, miR-548 was examined in a time-course experiment. In EV71-infected RD cells, miR-548 levels dropped sharply 6 h after infection and further decreased 12 h after infection (Fig. 6B). VSV infection also caused significant decrease in miR-548 levels at 6 h and 12 h after viral infection (Fig. 6C).

MiR-548 levels were suppressed and inversely correlated with IFN-λ1 in HBV patients

Our results showed that miR-548 mimics and inhibitors regulate IFN-λ1 expression and that endogenous expression of miR-548 was significantly decreased in response to virus infection. Thus, we sought to explore these results *in vivo* with a cohort of clinical samples collected from HBV patients and healthy individuals. Peripheral blood mononuclear cell (PBMC) infection by HBV was a frequent event among high viremia patients (Pasquinelli et al., 1986), and thus, we examined miR-548 expression in the PBMCs of HBV patients. The relative miR-548 levels in PBMC samples from HBV-infected patients (n = 17) were statistically lower than those from healthy individuals (n = 10, Fig. 7A). Upon examination of the IFN-λ1 mRNA levels in PBMCs, a statistically significant inverse correlation was observed between miR-548 and IFN-λ1 mRNA levels among the patients (n = 17, r = –0.703, P < 0.01, Pearson's correlation; Fig. 7B). Liver tissues from eight HBV-infected individuals were obtained from surgery and used for detection of miR-548 and IFN-λ1 mRNA. In these samples, a similar inverse correlation was observed between miR-548 and IFN-λ1 (n = 8, r = –0.733, P < 0.05, Pearson's correlation; Fig. 7C). Therefore, miR-548 appears to also regulate IFN-λ1 expression *in vivo* during viral infection.

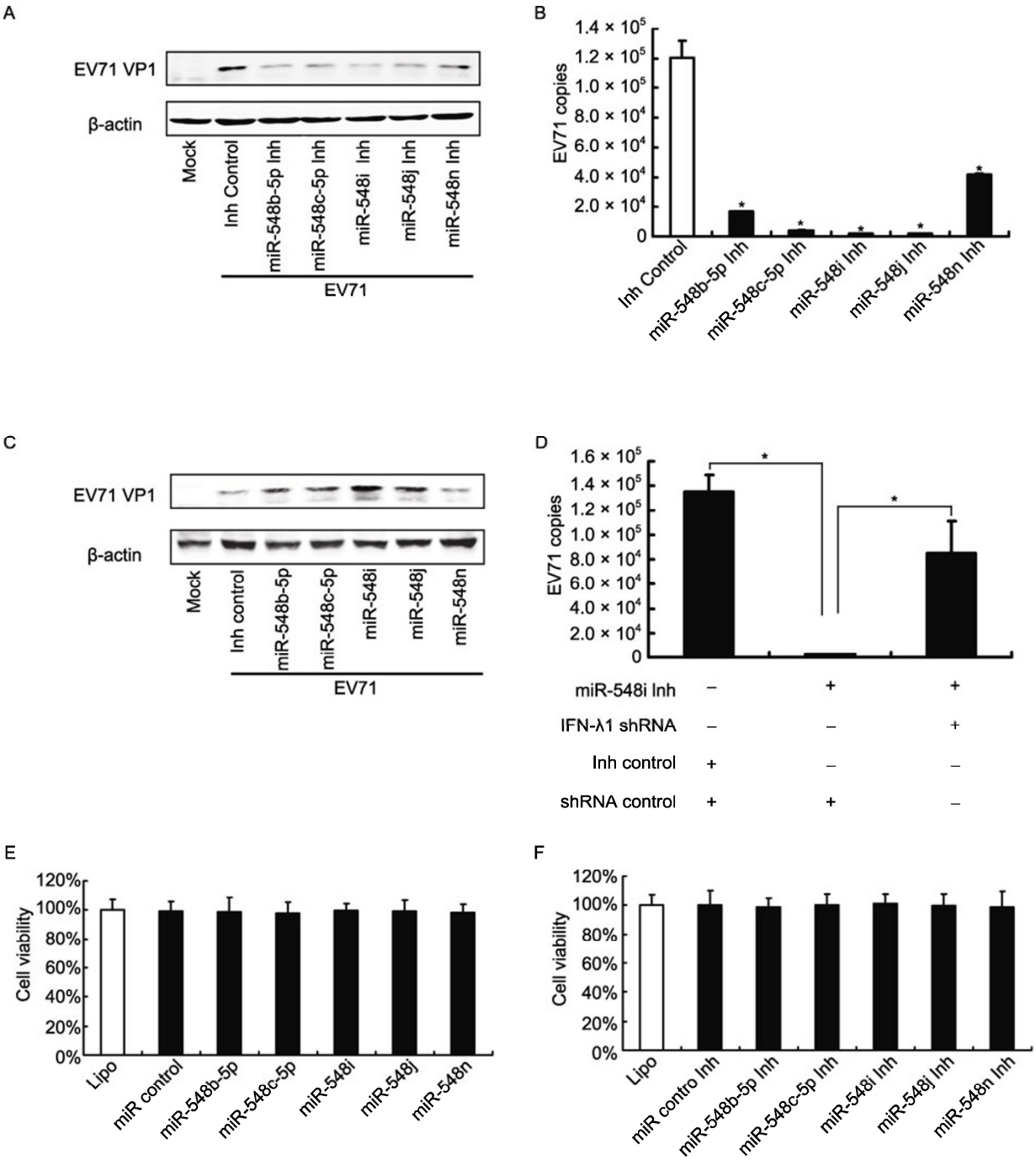

Figure 5. Antiviral assay following EV71 infection. (A) RD cells were transfected with each of the five subtypes of miRNA inhibitors or with a nonsense inhibitor control. Twenty-four hours later, the cells were infected with EV71 (MOI = 5) for 12 h. The EV71 VP1 protein was detected by western blot. Uninfected RD cells were used as a mock sample. (B) The culture supernatants from A were used to determine EV71 RNA copy. (C) RD cells were transfected with each of the five subtypes of miRNA mimics or a miRNA control for 24 h, followed by EV71 (MOI = 5) infection for 12 h. Western blot analysis was performed for EV71 VP1 protein. (D) miR-548i inhibitor, specific shRNA for IFN-λ1 or controls were transfected for 24 h. The antiviral assay as in A was performed and EV71 RNA copy was tested. (E and F) HepG2 cells were transfected with miR-548 mimics, inhibitors or controls, or treated with the Lipofectamine 2000 reagent only. MTT assays were performed 48 h after transfection. *$P < 0.05$.

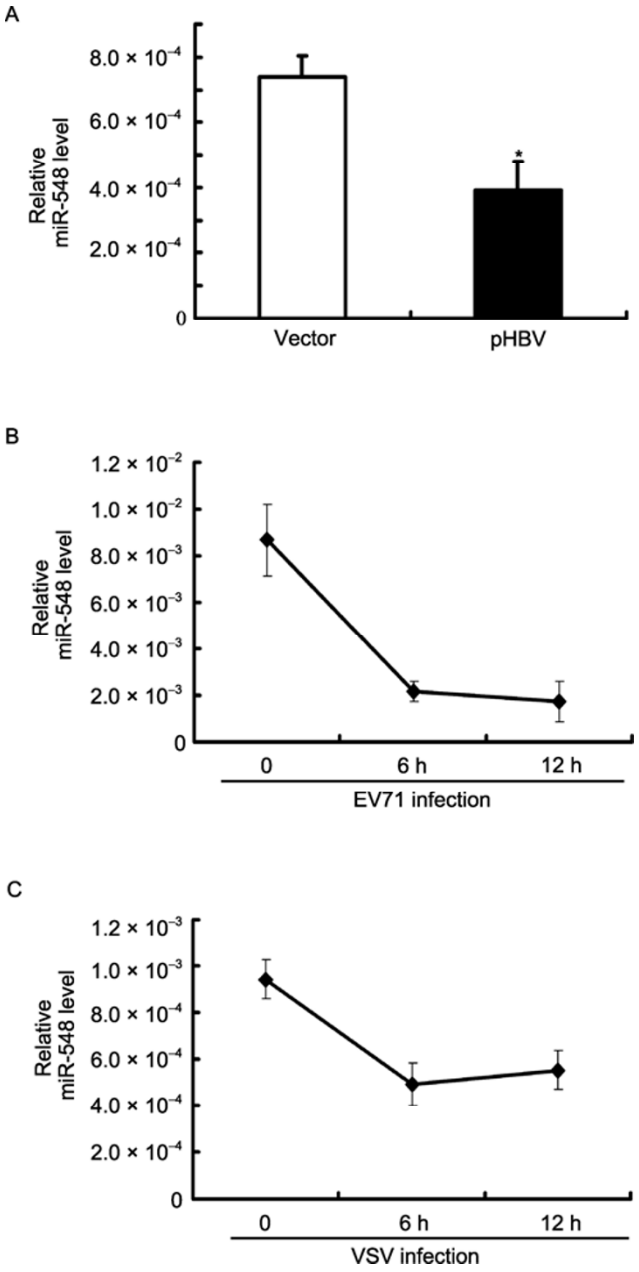

Figure 6. Suppressed miR-548 levels during viral infection. (A) HepG2 cells were tansfected with pHBV-1.3 or empty vector for 48 h, and relative miR-548 levels were detected by real-time PCR. (B) RD cells were infected with EV71 (MOI = 5) for 6 or 12 h, and then the relative miR-548 levels were analyzed by real-time PCR. Uninfected cells were used as 0 h samples. (C) An identical time couse for VSV infection was performed as in B. Heat inactivated EV71 or VSV were used as a negative control, and data were normalized to the value of the control. *P < 0.05.

DISSCUSION

In this study, we demonstrated that virus infection induced the activity of the IFN-λ1 3′UTR. Upon examination of the 3′UTR by miRNA prediction programs, several candidates of a primate-specific miRNA family were identified to be involved in this regulation. Indeed, Synthetic miR-548/inhibitors down-/up-regulate the activity of IFN-λ1 3′UTR, respectively. Additionally, miR-548 influenced the IFN-λ1 mRNA stability and the inhibition of endogenous miR-548 increased the

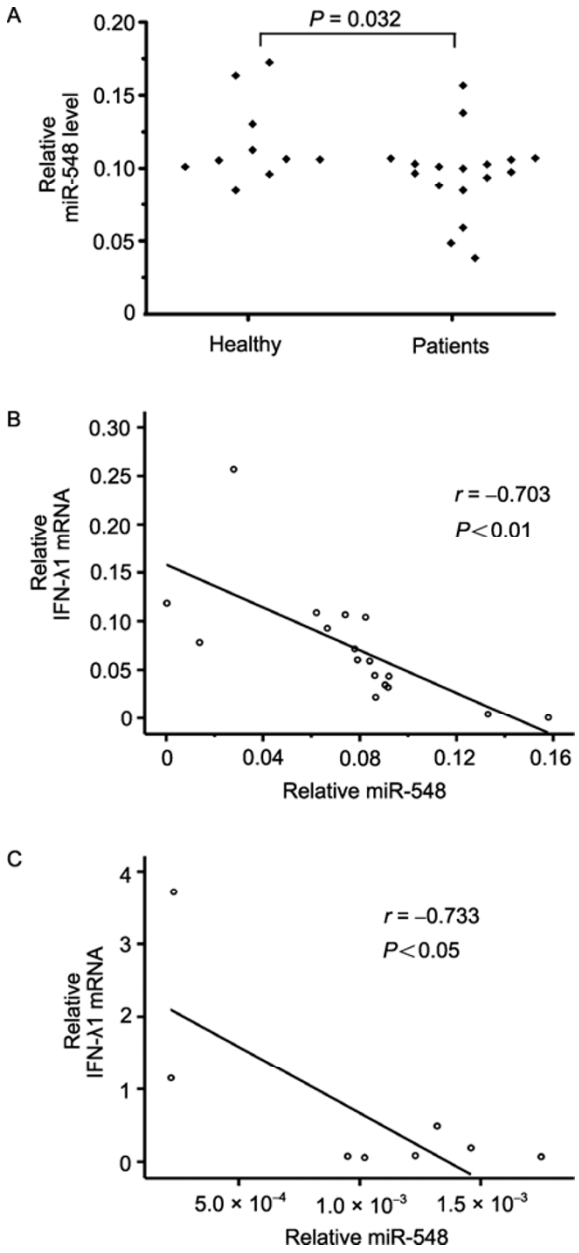

Figure 7. Analysis of clinical samples. (A) Relative miR-548 levels in PBMC samples from 10 healthy individuals and 17 hepatitis B patients were detected by real-time PCR and compared with the Student t-test. (B) Relative IFN-λ1 mRNA and miR-548 levels in 17 PBMC samples from hepatitis B patients were subjected to Pearson's correlation analysis. (C) The same analysis in B was performed with 8 HBV-infected liver tissue samples obtained from surgery. The "r" represented coefficient of Pearson's correlation.

mRNA level of IFN-λ1 (Fig. 3). The miR-548 family members affect the expression of IFN-λ1 via targeting the 3′UTR and regulate the IFN-λ1-mediated antiviral response (Figs. 2, 4 and 5). Suppression of endogenous miR-548 expression was observed in infected cells and in clinical samples infected with HBV (Fig. 6), and these findings allude to the participation of miR-548 in the natural process of infection. The IFN-λ1 levels were inversely correlated with miR-548 expression in patients (Fig. 7). Thus, we suggest that miR-548 regulates the antiviral response both *in vitro* and *in vivo*. Virus induces IFN-λ1 expression via a post-transcriptional process that involves suppression of miR-548 expression. This mechanism is a previously unrecognized mechanism for host antiviral response.

Five miRNAs (miR-548b-5p, miR-548c-5p, miR-548i, miR-548j, and miR-548n) were predicted to target IFN-λ1 3′UTR using prediction programs and confirmed using a 3′UTR targeting site test. Although the nucleotide sequences of these miRNAs are quite homologous, the activities of these miRNAs are different (Figs. 3–5). The miR-548i was the most effective regulator, and miR-548n was the least effective. According to the sequences shown in Fig. 2, these miRNAs, except miR-548n, were complementary to the 3′UTR of IFN-λ1 at their 5′-extremity. In agreement with our findings, we expected that the match of the 5′-terminus was important for the targeting efficiency and consequent activity. The 15th nucleotide (A) of MiR-548i was unique compared with the miR-548b-5p, miR-548c-5p, and miR-548j. Thus, the targeting efficiency may be strengthened via base pairing to the U-rich region in the IFN-λ1 3′UTR.

The biogenesis of miRNAs has been well characterized. Primary miRNA transcripts are first processed to produce a hairpin RNA of ~70 nt (Lee et al., 2003). Then, this pre-miRNA is exported into the cytoplasm via a pathway dependent on exportin-5 (Lund et al., 2004). In the cytoplasm, Dicer then cuts the hairpin to generate mature miRNAs (Grishok et al., 2001; Hutvagner et al., 2001). The mechanism of miRNA action, however, remains elusive. Evidence indicates that miRNAs cleave mRNAs with perfect complementarities (Hutvagner and Zamore, 2002) and repress the translation of mRNAs with imperfectly complementary binding sites (Saxena et al., 2003; Zeng et al., 2003). In our study, the miRNAs not only inhibited IFN-λ1 protein expression but also reduced the mRNA levels of IFN-λ1 (Fig. 3). In fact, such miRNAs that result in surprising reductions in mRNA levels by partial base pairing have been described previously. For example, miR-152 downregulated DNMT1 mRNA levels via partial binding of the mRNA 3′UTR (Huang et al., 2010). The same phenomenon was observed in the regulation of the cyclooxygenase-2 gene by miR-26b (Ji et al., 2010). In both cases, the inhibitors of the miRNA upregulated the mRNA levels of the respective target gene, and these findings resembled our results with the miR-548 inhibitors (Fig. 3A). This miRNA-induced decrease in mRNA levels via partially complementary binding to the 3′UTR cannot be explained with our current knowledge. Exhaustive research on the mechanism of miRNA action is required in the future.

In spite of the inexplicable mechanism, the fact that miR-548 inhibitors suppressed the levels of these miRNAs and resulted in the accumulation of the IFN-λ1 mRNA is quite interesting. Furthermore, the transfection of synthetic miR-548 inhibitors effectively suppressed viral infection with little influence on cell viability (Figs. 4 and 5), thus suggesting a potential mechanism for antiviral therapy.

MATERIALS AND METHODS

Clinical samples

Peripheral blood samples were obtained from 17 patients with chronic hepatitis B (10 males and 7 females with a mean age of 35.4 ± 8.2 y) and 10 healthy individuals (6 males and 4 females with a mean age of 35.7 ± 11.8 y). Eight HBV-infected tissues (paraneoplastic) were provided by Dr. Guozheng Yu (Zhongnan Hospital of Wuhan University). Total mRNA was extracted from PBMCs and liver tissues and then used for quantitative real-time PCR analysis. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Institutional Review Board of the College of Life Sciences, Wuhan University, in accordance with the guidelines for the protection of human subjects. Written informed consent was obtained from each participant.

Cell culture, transfection, and detection of IFN-λ1 protein

For cell culture, the human hepatoma HepG2 cell line was grown in DMEM medium, and the human embryonal rhabdomyosarcoma (RD) cells were grown in MEM medium. DMEM and MEM were purchased from Gibco-BRL (Gaithersburg, MD) and were supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were grown at 37°C in a 5% CO_2 incubator.

HepG2 or RD cells were plated in 24- or 6-well plates and grown to ~80% confluence at the time of transfection. The cells (2×10^5) were co-transfected with 0.2 μg DNA or 50 pmol of miRNA mimics/inhibitor, which were synthesized by Guangzhou RiboBio Co., Ltd. (China), using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The cells were serum starved for 24 h before harvesting.

The supernatents of virus-infected cells or control cells were used for IFN-λ1 detection using a commercial ELISA kit (eBioscience, San Diego, CA).

Construction of 3′UTR reporter plasmid and luciferase reporter assay

IFN-λ1 3′UTR-luc reporter plasmid was constructed by insertion of the 3′UTR into a vector as previously described (Yue et al., 2012) using primers P1–4 (Table S1). Following transfection of this plasmid, the induction of 3′UTR activity can be detected. A mutant construct was generated from the IFN-λ1 3′UTR reporter plasmid by utilizing the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA) using primers P5–6 (Table S1).

Each sample was co-transfected with the reporter plasmid and 50 ng of a pRL-TK plasmid expressing Renilla luciferase to monitor the

transfection efficiency (Promega, Madison, WI) as described previously (Li et al., 2008). Twenty-four hours after transfection, the cells were serum starved for an additional 24 h before harvest. The targeted luciferase activities were then measured and normalized to the Renilla luciferase activities. The assay results are expressed as relative luciferase activity.

Reverse transcription reaction and quantitative real-time PCR

Real-time PCR for mRNA was performed as described previously (Ank et al., 2006). The total RNA was extracted with the TRIzol reagent (Invitrogen), and the complementary DNA was generated with a reverse transcription system kit (Invitrogen). GAPDH was used as an endogenous control to normalize the amount of total mRNA in each sample. Stem-loop reverse transcription for mature miRNA was performed as previously described to produce the complementary DNA (Chen et al., 2005). U6 RNA was used as an miRNA internal control. Real-time PCR was performed with a standard SYBR-Green PCR kit protocol on a LightCycler 480 Ⅱ machine (Roche Applied Science, Indianapolis, IN). The primers (P7-20) used for IFN-λ1, MxA, OAS1, miR-548, and endogenous controls were listed in Table S1.

Viruses and measurement of viral replication and expression

The pHBV-1.3 plasmid was generated from the HBV genome (genotype D, GenBank accession no. U95551), digested with EcoRI/SalI, and inserted into pBluescript II. This plasmid was transfected into HepG2 cells to express HBV as previously described (Yue et al., 2012). HBeAg levels in the culture supernatants of pHBV-1.3-transfected cells were measured with commercial ELISA-based kits (Shanghai KeHua Biotech Co. Ltd., Shanghai, China).

EV71 (C4 subtype, Xiangfan, Hubei, China) was incubated with RD cells (MOI = 5) and grown on plates for 1 h with gentle shaking. The medium was removed, and the cells were washed twice with PBS and cultured in MEM. Following antiviral treatment, the RD cells were harvested for western blot analysis. The antibody used for EV71 VP1 detection was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To determine the number of copies of the viral mRNA, 100 μL of supernatant from the infected RD cells was dissolved in 1 mL TRIzol reagent, and the viral mRNA was extracted and reverse-transcribed into a cDNA library. Real-time PCR was performed using the VP1 gene of the EV71 construct plasmid at the standard concentration. The primers (P21-22) that were used are listed in Table S1.

Recombinant VSV expressing green fluorescent protein (VSV-eGFP) was a gift from Prof. Mingzhou Chen (Wuhan University, China). HepG2 cells were infected with VSV-eGFP at an MOI of 5. GFP expression directly reflects viral replication. GFP was visualized by fluorescence microscopy and analyzed by flow cytometry.

mRNA decay assay

The mRNA decay assay was performed as previously described (Strillacci et al., 2009). HepG2 cells were treated with poly I:C for 12 h. The cells were then transfected with synthetic miR-548i, shRNA-expressing plasmid or controls and followed by actinomycin-D (5 μg/mL) treatment. The relative IFN-λ1 mRNA levels were detected at 0, 12, 24, 36 and 48 h. GAPDH was used as an internal control. The IFN-λ1 protein level in the supernatant was detected with a commercial ELISA kit (eBioscience, San Diego, CA). Simultaneously, the cells at all the time points were collected and lysed into isochoric lysate. Isochoric lysate of each sample was used for western blot with β-actin antibody (Santa Cruz, CA). The bands were quantified with densitometric image analysis software and normalized the IFN-λ1 protein.

In vitro cytotoxicity assays

The cytotoxicity of miR-548 mimics and inhibitors was tested in HepG2 cells using the MTT assay as described previously (Yang et al., 2012). Briefly, cells were seeded in a 96-well plate at a density of 4000 cells/well and allowed to adhere for 24 h prior to the assay. Cells were transfected with synthetic miR-548 mimics and inhibitors or nonsense control miRNAs, and samples transfected with only Lipofectamine 2000 reagent were used as a control. After 48 h of transfection, 20 μL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoniumbromide) solution (5 mg/mL) was added to each well of the plate. After a 4-h incubation, 200 μL/well of DMSO was added to dissolve the contents of the plate, and the absorbance of the obtained DMSO solution was measured at 570 nm and 630 nm by a microplate reader (ELx800, Bio-Tek Inc., Winooski, VT).

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ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; EV71, enterovirus-71; GAS, gamma activated sequence; GFP, green fluorescence protein; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN-α receptor; IRF, interferon regulatory factor; IL, interleukin; ISG, interferon stimulated gene; ISRE, IFN stimulated response element; JAK, Janus tyrosine kinase; miR, miRNA, microRNA; MITEs, miniature inverted-repeat transposable elements; MOI, multiplicity of infection; mRNA, messenger RNA; MxA, the human myxovirus resistance protein 1; NF-κB, nuclear factor-kappa B; OAS, oligoadenylate synthetase; PBMC, peripheral blood mononuclear cell; RD, human embryonal rhabdomyosarcoma; RIG-I, retinoid acid-inducible gene I; shRNA, short hairpin RNA; STAT, signal transducers and activators of transcription; TLR, toll-like receptor; UTR, untranslated region; VISA, virus-induced signaling adapter; VSV, vesicular stomatitis virus

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