Human MicroRNA-602 Inhibits Hepatitis C Virus Genotype 1b Infection and Promotes Tumor Suppressor Gene Expression in a Hepatoma Cell Line

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Abstract

Objective: Hepatitis C virus (HCV) infects 200 million people worldwide, inducing cirrhosis and hepatocellular carcinoma. Liver-specific microRNA (miR) miR-122 facilitates HCV replication, and Locked Nucleic Acid (LNA)-anti-miR-122 was used successfully as a mono-therapy in HCV infected patients. Studies show that anti-miR-122 may negatively impact hepatocyte metabolism. Previously, we showed that human (hsa) miR-602 relative intracellular expression was up-regulated in microarray profiling when transfected with HCV genotype 1b, and hsa-miR-602 lowered HCV accumulation in infected Huh-7.5 cells. This study compares the effectiveness of hsa-miR-602 and LNA-anti-miR-122 on inhibiting HCV replication and regulating the expression of tumor suppressor genes p53, p73, and RASSF1A.

Methods: Huh-7.5 cells were transfected with HCV genotype 1b and treated with hsa-miR-602, LNA-anti-miR-122 or both. Cytopathic effect (CPE) and cell aggregation were observed regularly after transfection. HCV load and localization in the un-transfected, transfected, and treated cells were detected by qRT-PCR and immunostaining, respectively. qRT-PCR detected the expression of p53, p73, and RASSF1A. The Cancer Cell Lines Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) were used to correlate gene copy number and expression for TP53, TP73 and RASSF1A.

Results: HCV load decreased in all treated cells but was more pronounced with hsa-miR-602. HCV transfected Huh-7.5 cells treated with hsa-miR-602 showed no morphological changes whereas cells treated with LNA-anti-miR-122 or LNA-anti-miR-122 and hsa-miR-602 together showed morphological changes. p53, p73, RASSF1A were down-regulated in HCV transfected cells and all treatments restored expression to near normal levels. Similar down-regulated expression was seen in the HCC patient samples and cell lines.

Conclusion: Hsa-miR-602 effectively reduces HCV load and restores p53, p73, and RASSF1A expression in HCV transfected cells.

Introduction

Hepatitis is an inflammation of the liver. It is commonly caused by toxins, certain medications, drugs, autoimmune diseases, alcohol and viral infections. Common symptoms for hepatitis include decreased appetite, abdominal pain, dark urine, pale-colored stools, joint pain, unexplained weight loss and jaundice. Viral hepatitis is classified into five types, as hepatitis A, B, C, D and E. Hepatitis C Virus (HCV) is a small (50 nm in size) enveloped positive sense single-stranded (ss) RNA virus. The genome of HCV has a single open reading frame of 9,600 nucleotide bases long (9.6 kb). It belongs to Hepacivirus genus in the family Flaviviridae. Sequence analyses of HCV isolates around the world have revealed the presence of six major genotypes, 1 through 6, and subtypes, a through r [1]. About 200 million people or three percent of the world’s population are infected with HCV. Three to four million people are newly infected with HCV each year, with 170 million chronic carriers at risk of developing liver cirrhosis or liver cancer [2]. Globally, the most common types of hepatitis viruses are hepatitis A (HAV), hepatitis B (HBV), and hepatitis C (HCV). Approximately 5.2 million people in the United States have chronic HCV infection [3]. Hepatitis C starts as an acute infection and can progress to chronic infection after six months if not resolved. Chronic hepatitis leads to the following subsequent conditions: fibrosis, cirrhosis, liver failure, hepatocellular carcinoma (HCC) and death. Objective studies suggest that a substantial proportion of global health problems associated with liver diseases worldwide are attributed to HCV infection.

There are vaccines available to prevent HAV and HBV infections. Unfortunately, there is no vaccine currently available to prevent HCV infection. According to CDC, the estimated actual cases in the United States for 2013 for HAV, HBV and HCV are 3,500, 19,800 and 29,700, respectively. The number of deaths that occurred in 2013 in the United States due to HAV, HBV and HCV are 80, 1873 and 19,368, respectively [4]. Considering the 2013 U.S. death rates attributed to HCV, an effective treatment is needed to prevent HCV infection. Patients with chronic HCV infection are administered standard therapy that consists of a combination of pegylated interferon-α (PEG-IFN-α), ribavirin (RBV), and protease inhibitors boceprevir and telaprevir [5]. The first-line option in the treatment for HCV genotype 1 infected patients with advanced fibrosis and cirrhosis is currently triple therapy with boceprevir/ telaprevir and pegylated interferon-ribavirin. This triple therapy is effective in 50% to 70% of patients with CHC [6]. Clinical cure with a combination of PEG-IFN-α and RBV therapy is possible, with 40% to 50% of genotype 1-infected patients and 70% to 80% of genotypes 2/3-infected patients benefiting from this treatment. Other patients either experience relapse, or they neither qualify, nor tolerate the standard therapy [7-9], which is due to the genetic diversity of HCV genotypes and genetic variation in host factor interleukin 28B (IL28B) [10]. Presently, there are a number of recommendations for HCV treatment for all genotypes by the American Association for the
Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA), such as sofosbuvir/ledipasvir [11], simprevir [12], sofosbuvir [12] and Viekira Pak [13]. The preferred PEG-IFN-α free regimens for treating HCV genotype 1b infection are sofosbuvir/ledipasvir, or simprevir and sofosbuvir or Viekira Pak for 12 weeks in cases of no cirrhosis. Simprevir and sofosbuvir are used for 24 weeks or Viekira Pak in combination with ribavirin for 12 weeks in cases of cirrhosis [14-16]. Most of these treatments are administered with ribavirin, which is associated with adverse events [17], requires longer treatment duration, and are expensive.

MicroRNAs (miRNAs) are an abundant class of small endogenous non-coding RNAs, approximately 22 nucleotides (nt) in length that direct post-transcriptional regulation of gene expression. MicroRNA-122 (miR-122) represents approximately 50% of the total miRNA population expressed in human liver [18], and has also been suggested to be important for maintaining liver cell identity. MiR-122 has four binding sites in the HCV genome [19] and has been implicated in fatty acid and cholesterol metabolism and HCV infection [20-23]. Since the first publication reporting that miR-122 facilitates HCV replication in human liver [20], this liver-specific miRNA has become a focus of numerous research projects investigating the interaction between liver cells and HCV, and the possible role of miR-122 as a target for antiviral intervention [20]. MiRNA inhibitors are chemically modified, single stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA. The miR-122 inhibitor, miravirsen, is a 15 nt locked nucleic acid (LNA) high-affinity RNA analog (LNA-anti-miR-122 or SP63649) that is complementary to miR-122, in which the ribose ring is “locked” in the ideal conformation for Watson-Crick binding, exhibiting thermal stability and specificity when hybridized to a complementary DNA or RNA strand. Chronically infected chimpanzees with HCV genotype 1 when treated with miravirsen experienced long-lasting suppression of HCV without any evidence of side effects [23]. Miravirsen was used as a monotherapy treatment in phase 2a clinical trials in patients with HCV genotype 1 infection. Results from the study showed dose-dependent anti-viral activity. It was also observed that the inhibition of miR-122 in HCV patients resulted in decreased levels of serum cholesterol homeostasis, consistent with previous studies in mice and primates [21-23]. The most common adverse events observed were headache, fatigue, nasopharyngitis, nausea, rash, diarrhea, myalgia, pruritus and flu-like symptoms [24].

Previously we have reported that human microRNA-602 (hsa-miR-602) which was up-regulated 1256.32 fold in microarray profiling, when transfected with HCV genotype 1b RNA in Huh-7.5 hepatoma cells, decreased HCV accumulation in the infected Huh-7.5 cells [25]. In the present study, we examined HCV load and expression of the tumor suppressor genes p53, p73 and RASSF1A in Huh-7.5 cells transfected with HCV genotype 1b RNA and then treated with c-hsa-miR-602 (cloned in pLentiVirus), LNA-anti-miR-122, or combination of c-hsa-miR-602 and LNA-anti-miR-122. Our results show that hsa-miR-602 reduces Hepatitis C Virus genotype 1b load and promotes p53, p73, and RASSF1A expression in a hepatoma cell line.

Materials and Methods

Plasmid: Plasmid KT-9 (HCV genotype 1b) [26] was used in this study because HCV genotype 1b infection is globally prevalent [27].

In vitro RNA transcription and cDNA synthesis

Plasmids carrying KT-9 constructs were linearized with XbaI and plasmid DNA was purified with the QIAquick Gel Extraction Kit protocol (Cat # 28704, Qiagen, GmbH, Hilden). Purified DNA was then subjected to an in vitro transcription reaction using MEGAscript T7 RNA polymerase (Cat # AM1334, Ambion, TX) according to the manufacturer’s instructions. RNA from the in vitro transcription reaction was purified with the NucleoSpin® RNA II kit (Cat # 740955.50, Macherey-Nagel, GmbH, Düren). RNA quantitation was achieved by measuring the optical density at 260 nm using NanoDrop Lite. cDNA was synthesized using an iScript cDNA Synthesis Kit (Cat # 170-8890, BIO-RAD, Hercules, CA), according to manufacturer’s instructions.

HCV genotype 1b nested-PCR amplification

In order to confirm HCV genotype 1b, a nested PCR was carried out as described previously [28] using the primer pair HCV outer sense and HCV outer antisense (Table 1) from the 5′ NCR (non-coding region) for the first round of PCR. One microoliter of the first round PCR product was amplified with HCV genotype 1b nested inner sense primer and HCV genotype 1b antisense primer (Table 1). Amplified products were separated by 2% agarose gel electrophoresis and viewed under the UV transilluminator. First round PCR and nested PCR produced amplification products of 470 bp and 233 bp, respectively [25], which are specific for HCV genotype 1b.

Cell line and cell culture

Huh-7.5 cells are a sub-line derived from Huh-7 hepatoma cell line cured of the HCV replicon with interferon and are highly permissive for the initiation of HCV replication [29]. Huh-7.5 cells were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM, Cat # D5796, Sigma-Aldrich, Saint Louis, MO). The cells were cultured and prepared for transfection as previously described [25].

Human miR-602

Hsa-miR-602 was obtained from Applied Biological Materials Inc (Richmond, BC, Canada) and was cloned in pLentiVirus vector

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>HCV outer sense</td>
<td>5′-TTGTTGCTACTGCTGTAGG3′</td>
</tr>
<tr>
<td>HCV outer antisense</td>
<td>5′-GGATGTACCCCATGAGGTCG3′</td>
</tr>
<tr>
<td>HCV genotype 1b nested inner sense primer</td>
<td>5′-GTGCCCGGGAGGAGGTCTCAG3′</td>
</tr>
<tr>
<td>HCV genotype 1b antisense primer</td>
<td>5′-AGCCCTGGGATAGTTGTC3′</td>
</tr>
<tr>
<td>Forward miR primer</td>
<td>5′-CTCGGCAATGGAGACGGTACAG3′</td>
</tr>
<tr>
<td>Reverse miR primer</td>
<td>5′-TGGAATAGCTCACAGGCAGGCACG3′</td>
</tr>
<tr>
<td>HCV TAQ1</td>
<td>5′-GCTCTACATGCTGGGATAGTA3′</td>
</tr>
<tr>
<td>HCV TAQ 2</td>
<td>5′-GTACTCAAGGGTTCCCAGC3′</td>
</tr>
<tr>
<td>Forward p53</td>
<td>5′-AAGCCGTATCCGGGACCAC3′</td>
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<tr>
<td>Reverse p53</td>
<td>5′-CGTGATACGGTGTCGGA3′</td>
</tr>
<tr>
<td>Forward p73</td>
<td>5′-AAGCCGTCCCTCAACACGAG3′</td>
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<tr>
<td>Reverse p73</td>
<td>5′-GGCCTGCTGGGTCCTCAAC3′</td>
</tr>
<tr>
<td>Forward RASSF1A</td>
<td>5′-CGTTGATACGGGTCGTT3′</td>
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<tr>
<td>Reverse RASSF1A</td>
<td>5′-CCGGAGTCCTCCGTTAAACG3′</td>
</tr>
<tr>
<td>Forward β-actin</td>
<td>5′-TGGTTGCTACTGCTGTAGG3′</td>
</tr>
<tr>
<td>Reverse β-actin</td>
<td>5′-AACCAATAAACACTCTCCTCCCTTA3′</td>
</tr>
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Table 1: PCR primers used for this study.
(c-hsa-miR-602) which has Green Fluorescent Protein (GFP) and puromycin resistant selection markers.

**Amplification of hsa-miR-602 from pLentivirus vector:** PCR was used to confirm the presence of hsa-miR-602 in pLentivirus with Forward miR primer and Reverse miR primer (Table 1). Amplified fragments were separated by 2% agarose gel electrophoresis and viewed under the UV transilluminator. pLentivirus without and with hsa-miR-602 yielded an amplified product of 407 bp and 907 bp, respectively (data not shown).

**HCV genotype 1b RNA transfection:** In vitro transfection was carried out according to the protocol described earlier [25]. The transfected and un-transfected cells were incubated overnight at 37°C in 5% CO₂. The next day the media were changed with pre-warmed complete DMEM. All of the Huh-7.5 cells were allowed to grow for 10 days at 37°C in 5% CO₂ until 80-90% confluency was obtained.

**Detection of HCV in infected Huh-7.5 cells by quantitative Reverse Transcripase-PCR (qRT-PCR):** RNA was isolated with miRNeasy Mini Kit (Cat # 217004, Qiagen, GmbH, Hilden), according to the manufacturer’s protocol. The presence of HCV RNA (Ct<33) in transfected and un-transfected Huh-7.5 cells, using iScript™ One-Step RT-PCR Kit with SYBR® Green (Cat # 170-8893, BIO-RAD, Hercules, CA) and HCV primers HCV TAQ1 and HCV TAQ 2 (Table 1) [30]. The HCV primer sequences were directed against the 5' NCR of the HCV genome. Amplification was performed according to the protocol described earlier [25]. qRT-PCR reactions were run in triplicate.

**Treatment of HCV infected Huh-7.5 cells with c-hsa-miR-602, LNA-anti-miR-122, and combination of c-hsa-miR-602 and LNA-anti-miR-122:** The Huh-7.5 cells transfected with HCV genotype 1b RNA and their respective un-transfected cells were then treated on day 10 with: 1.0 µg of pLentivirus, 1.0 µg of c-hsa-miR-602, 25 nM of Locked Nucleic Acid (LNA) anti-miR-122 or with a combination of c-hsa-miR-602 and LNA-anti-miR-122 (Table 2) for 24 hrs using Lipofectamine 2000 reagent (Cat # 11668-019, Invitrogen, Carlsbad, CA), stored for RNA isolation and used for GFP fluorescence and HCV localization. All experiments were performed twice and ran in triplicates.

**Observation of cytopathic effect:** Transfected, un-transfected and treated Huh-7.5 cells were observed regularly for formation of cytopathic effects (CPE).

**Selection of Huh-7.5 cells, Green Fluorescent Protein (GFP) fluorescence and HCV localization by immunostaining in transfected and un-transfected Huh-7.5 cells:** After 8 days of treatment with c-hsa-miR-602, LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602, Huh-7.5 cells were washed with 1X PBS, trypsinized, and centrifuged, and the cell pellets were re-suspended in complete DMEM. The cells were added to their respective labeled 8 well chamber slides (Cat # T-2820-8, Nunc® Lab-Tek II Glass Chamber Slide”, Thermo Scientific, Wilmington, DE) and were checked for GFP fluorescence to see the efficiency of transfection and HCV localization with E2 antibody (H52) [31] as described previously [25]. After GFP efficiency was checked, the un-transfected and transfected cells were added to their respective labeled flask with complete DMEM and selection medium (complete DMEM containing 0.5 µg/mL of puromycin [Cat # P9620-10ML, Sigma Aldrich, St. Louis, MO]), respectively, (Table 2) and incubated at 37°C in 5% CO₂ incubator. GFP and HCV localization were observed at 4, 8, 12, and 16 weeks.

Huh-7.5 cells treated with pLentivirus, c-hsa-miR-602 or in combination with LNA-anti-miR-122 with and without HCV genotype 1b RNA, were subjected to selection medium for 12 weeks. After 12 weeks of selection, the selection medium was replaced with complete DMEM in Huh-7.5 cells and incubated at 37°C in 5% CO₂ for additional four weeks. Un-transfected Huh-7.5 cells, Huh-7.5 cells transfected with HCV genotype 1b RNA and Huh-7.5 cells treated with LNA-anti-miR-122 with and without HCV genotype 1b RNA were not subjected to selection medium because no pLentivirus with puromycin selection marker was added. Each week, cells were passaged, preserved in recovery cell culture freezing medium (Cat # 12648-010, Life Technologies, Carlsbad, CA), stored for RNA isolation and used for GFP fluorescence and HCV localization. All experiments were performed twice and ran in triplicates.

**HCV RNA isolation from transfected, un-transfected and treated Huh-7.5 cells:** RNA from transfected, un-transfected, and treated and Huh-7.5 cells were isolated with miRNeasy Mini Kit (Cat # 217004, Qiagen, GmbH, Hilden), according to the manufacturer’s protocol and quantified using NanoDrop Lite.

**Detection of HCV load in transfected, un-transfected and treated Huh-7.5 cells by Quantitative Reverse Transcrptase-PCR (qRT-PCR):** The iScript™ One-Step RT-PCR SYBR® Green kit was
used (Cat# 170-8893, BIO-RAD Laboratories, Hercules, CA) to perform the qRT-PCR to detect the HCV load and the expression of three tumor suppressor genes (p53, p73 and RASSFA) from transfected and un-transfected cells before and after puromycin selection at 4, 8, 12, and 16 weeks. Master mix consisted of 2 X SYBER Green RT-PCR reaction mix of HCV primers (HCV TAQ1 and HCV TAQ 2) (Table 1) [36], p53 primers (Forward p53 and Reverse p53) (Table 1) [32], p73 primers (Forward p73 and Reverse p73) (Table 1) [33], RASSFA1 primers (Forward RASSFA1 and Reverse RASSFA1) (Table 1) [34], or β-actin primers (Forward β-actin and Reverse β-actin) (Table 1) [34]. One-Step RT-PCR was performed as described previously [25], β-actin, a house-keeping gene (HKG), was used to obtain a normalized Ct value for both experimental and control samples. Expression of HCV and tumor suppressor genes was determined for the normalized experimental samples to their respective controls (Table 2) using 2^(ΔΔCt) method. All experiments were performed twice and ran in triplicate.

Statistical analysis

Gene correlation analysis between gene copy number and the corresponding gene expression for TP53, TP73 and RASSFA1 were conducted using data obtained from The Cancer Cell Lines Encyclopedia (CCLE) (http://www.broadinstitute.org/ccle) and The Cancer Genome Atlas (TCGA) (http://www.cbioportal.org/publicportal/). Statistical analysis was conducted using program (https://cran.r-project.org/ ) and survival curve for patients with MIR-602 expression were done using TCGA hepatocellular carcinoma (HCC) data through UCSC cancer genome browser.

Results

c-hsa-miR-602 and LNA-anti-miR-122 reduces cytopathic effect and cell aggregates in HCV transfected Huh-7.5 cells

To determine the effect of c-hsa-miR-602, and LNA-anti-miR-122 in Huh-7.5 cells transfected with HCV genotype 1b RNA, cells were treated with c-hsa-miR-602, LNA-anti-miR-122 or in combination. Un-transfected Huh-7.5 cells showed neither CPE and cell aggregate nor any morphological changes over 16 weeks (Figure 1A). The Huh-7.5 cells transfected with HCV genotype 1b RNA showed few aggregates of cells and CPE formation at day 4 of transfection. The CPE and cell aggregates increased over 16 weeks, indicating spread of infection (Figure 1B). Huh-7.5 cells treated with pLentivirus or c-hsa-miR-602 showed only CPE formation before and after puromycin selection and no change in cell morphology over 16 weeks (Figures 1C and 1D, respectively). It was also observed that Huh-7.5 cells transfected with HCV genotype 1b RNA and then treated with pLentivirus showed CPE and cell aggregation over the 16 weeks but no changes in cell morphology were observed (Figure 1E).

Huh-7.5 cells transfected with HCV genotype 1b RNA and then treated with c-hsa-miR-602 showed a decrease in CPE and cell aggregation at 8 weeks of treatment (data not shown) and no change in cell morphology over 16 weeks. The CPE decreased with time and no cell aggregates were observed at 12 weeks (data not shown) and 16 weeks (Figure 1F). Whereas, Huh-7.5 cells transfected with HCV genotype 1b RNA and then treated with LNA-anti-miR-122, or LNA-anti-miR-22 in combination with c-hsa-miR-602 showed a decrease in CPE and cell aggregation at 4 weeks of treatment that diminished further at 8 weeks and 12 weeks of treatment with slight changes in cell morphology observed at 12 weeks of treatment with slight changes in cell morphology observed at 12 weeks. The morphological changes due to LNA-anti-miR-122 treatment observed at 12 weeks in Huh-7.5 cells transfected with HCV genotype 1b RNA increased substantially with time (Figure 1G: 16 weeks). Whereas, Huh-7.5 cells transfected with HCV genotype 1b RNA and treated with LNA-anti-miR-122 in combination with c-hsa-miR-602 showed some morphological changes at 12 weeks which increased slightly with time when compared to LNA-anti-miR-122 alone (Figure 1H: 16 weeks). Huh-7.5 cells only treated with LNA-anti-miR-122 (Figure 1I: 16 weeks) and LNA-anti-miR-122 in combination with c-hsa-miR-602 (Figure 1J: 16 weeks) but not transfected with HCV genotype 1b RNA also showed morphological changes and little CPE but no cell aggregation were seen. These results clearly indicate that c-hsa-miR-602 reduces CPE and cell aggregation without inducing any morphological changes in Huh-7.5 cells.

c-hsa-miR-602 and LNA-anti-miR-122 reduces HCV infection:

To observe the efficacy of transfection and HCV localization, un-transfected and transfected Huh-7.5 cells were examined for GFP fluorescence and stained with E2 antibody (H52), respectively. The un-transfected Huh-7.5 cells showed neither GFP fluorescence nor HCV localization (red punctate dot-like structures) over 16 weeks of incubation (Figure 2A). It was also observed that all of the Huh-7.5 cells treated with pLentivirus, c-hsa-miR-602, or LNA-anti-miR-122 in combination with c-hsa-miR-602 showed GFP fluorescence at 4 weeks through 16 weeks (data not shown). Huh-7.5 cells treated with only LNA-anti-miR-122 showed no GFP fluorescence because LNA-anti-miR-122 was not cloned into a vector containing the GFP marker (data not shown). Also all these treated but un-transfected Huh-7.5 cells did not show HCV localization.

Huh-7.5 cells transfected with only HCV showed an increase...
in an average number of red punctate dot-like structures at 8, 12 and 16 weeks (Figure 2B) ranging from 73.33%, 83.33% and 90%, respectively. The Huh-7.5 cells that were transfected with HCV and treated with pLentivirus showed GFP fluorescence and an increase in the average number of red punctate dot-like structures at 8, 12 and 16 weeks (Figure 2C) ranging from 70%, 76.66% and 86.66%, respectively. Huh-7.5 cells transfected with HCV and treated with c-hsa-miR-602 showed GFP fluorescence and a decrease in red punctate dot-like structures at 8, 12 and 16 weeks (Figure 2D) ranging from 53.33%, 33.33% and 16.66%, respectively. The number of red punctate dot-like structures decreased at 4 weeks to 16 weeks (Figure 2D) in the cells transfected with HCV and treated with only LNA-anti-miR-122 from 40% to 16.66%, respectively. Huh-7.5 cells transfected with HCV and treated with LNA-anti-miR-122 in combination with c-hsa-miR-602 showed GFP fluorescence and decrease in the number of red punctate dot-like structures from 33.33% to 10% at 4 weeks to 16 weeks, respectively, (Figure 2F). The immunostaining results indicate that LNA-anti-miR-122 treatment in combination with c-hsa-miR-602 is effective in decreasing the localization of HCV in Huh-7.5 cells.

c-hsa-miR-602 decreases HCV load and up-regulates the expression of tumor suppressor genes p53, p73 and RASSF1A in transfected Huh-7.5 cells: qRT-PCR was used to evaluate the effect on HCV load and expression of tumor suppressor genes p53, p73 and RASSF1A in Huh-7.5 cells transfected with HCV and treated with c-hsa-miR-602, LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602. It was observed that Huh-7.5 cells transfected with HCV and treated with pLentivirus showed a gradual down regulation of the three tumor suppressor genes, and a gradual increase in HCV load over the course of 16 weeks. Huh-7.5 cells that were transfected with HCV and treated with c-hsa-miR-602, LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602, in initial stages of infection showed down regulation in the three tumor suppressor genes at 4 weeks. However, at 8, 12, and 16 weeks, all the tumor suppressor genes approached the normal expression level, and there was a decrease in HCV load. It was also observed that Huh-7.5 cells transfected with HCV and treated with c-hsa-miR-602 lowered the HCV load by 2.5 fold or 2.3 fold at 16 weeks when compared to LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602, respectively (Figures 3A-D). The qRT-PCR results indicate that c-hsa-miR-602 treatment is effective in decreasing the HCV load and increases the expression of p53, p73 and RASSF1A in HCV transfected Huh-7.5 cells. The decrease in viral load is consistent with immunostaining showing reduced localization of HCV with E2 antibody (H52).

Expression of tumor suppressor genes and MIR-602 from HCC clinical studies

Our data indicates that c-hsa-miR-602 treatment increases p53, p73, RASSF1A expression reducing HCV infection. It is well documented that HCV is a major risk for the development of HCC. To investigate the genetic alterations of these tumor suppressor genes in HCC, we first conducted the oncprint analysis for TCGA HCC tissue samples (N=424). We found that 33%, 3% and 2% of the samples had genetic alterations in TP53, TP73, and RASSF1, respectively, where the most dominant modification was deep deletion and missense mutation (Figure 4A). The mRNA expression level between normal and HCC patient samples for three tumor suppressor genes were significantly down regulated (Figures 4B-D) which is consistent with our in vitro findings (Figures 3A-D). We further studied the regression correlation analysis between tumor suppressor gene copy number alteration (CNA) and miRNA expression and revealed that all three genes are significantly correlated with their respective gene CNA and miRNA expression as well as correlated to loss of function mutations in most HCC samples (Figures 4E-G).

We also analyzed the HCC cell line (N=27) model data from The Cancer Lines Encyclopedia (CCLE) and found that most HCC cell lines show down regulation of TP53, TP73, and RASSF1 (Figure 5A). Gene expression correlation analysis using agglomerative clustering based heat map analysis for copy number expression also revealed that the tumor suppressor genes have either loss of copy number or low copy number expression in most of the HCC cell lines (Figure 5B). The Pearson correlation coefficient was also calculated between tumor suppressor gene CNA and corresponding mRNA expression and revealed that a significant correlation existed between CNA and mRNA expression, and loss of function mutations in most HCC cell lines (Figures 5C-E).

Interestingly, up-regulation of MIR-602 was found significantly correlated with a higher rate (63.71%) of overall patient survival (Figure 6) and suggest that MIR-602 can be a prognostic marker for HCC. The cohort table containing TCGA HCC patient survival datasets are available in the Supplementary Table 1.

Discussion

Hepatitis C is a serious liver disease worldwide caused by HCV and has stimulated research into developing strategies for the screening, diagnosis, prevention and treatment of HCV associated diseases. Several new treatments including Harvoni, Olysio and Sovaldi, Viekira Pak, Sofosbuvir-based regimens have been approved by the Food and Drug Administration (FDA) providing more options for treatment for CHC patients. These new treatments are not only expensive, e.g., market pricing of a 12-weeks course of sofosbuvir is approximately US $84,000 [6], but also have side effects, such as: tiredness, headache, rash, itching, nausea, muscle pain, indigestion,
insomnia, decreased appetite and diarrhea [11,12]. These factors have prompted scientists to find alternate medical therapies using RNAi for treating HCV infection.

Many miRNAs play a role in controlling a variety of biological functions, including cell differentiation, cell proliferation, developmental patterning and transcriptional regulation [35]. Experimental evidence supports the idea of aberrant miRNA expression in the development of various human malignancies, showing that different sets of miRNAs are usually deregulated in different cancers and can represent a promising new class of cancer biomarkers [36,37]. Viruses involved in carcinogenesis have also been found to encode miRNAs [38]. MiR-122 is essential for HCV replication, and data indicate that it also serves as a viable therapeutic target [39,20,23]. However, miR-122 is essential in maintaining hepatic phenotype and its loss of function causes hepatocarcinogenesis in mice [40] suggesting that the long-term depletion of miR-122 may have deleterious consequences in humans. Both miR-602 and miR-122 are expressed in human liver. MiR-122 is the most highly expressed miRNA in human hepatocytes [18]. The common models used for HCV in vitro study are Huh-7.5 and Huh-7 cells [41,42].

In our previous study, we showed that the intracellular expression of hsa-miR-602 in Huh-7.5 cells transfected with HCV genotype 1b was low [25], which was up-regulated 1256.32 fold in microarray profiling when transfected with HCV genotype 1b and then treated with c-hsa-miR-602. LNA-anti-miR-122 alone or in combination with c-hsa-miR-602 showed down regulation in the three tumor suppression genes, before selection (BS) (day 18 of transfaction with HCV). From 4 weeks onwards, the expression for the three tumor suppressor genes started to move towards normal levels, and also displayed a decrease in HCV genotype 1b load. It was also observed that Huh-7.5 cells that were transfected with HCV genotype 1b and treated with c-hsa-miR-602 alone, showed a gradual decrease in HCV load when compared to LNA-anti-miR-122 alone or in combination with c-hsa-miR-602.

Figure 3: Effect of c-hsa-miR-602 and LNA-anti-miR-122 on HCV genotype 1b load and p53, p73 and RASSF1A expression over 16 weeks in Huh-7.5 cells by qRT-PCR. (Figures 3A-D). Data are shown as log_{10} ratio and reflect the expression of HCV genotype 1b and tumor suppressor genes p53, p73 and RASSF1A in Huh-7.5 cells transfected with HCV genotype 1b and treated with pLentivirus, c-hsa-miR-602, LNA-anti-miR-122 alone or in combination with c-hsa-miR-602. Expression of HCV, p53, p73 and RASSF1A were normalized using the β-actin expression level as a HKG. The normalized experimental values were analyzed with their respective normalized control values using the 2^{-ΔΔCt} method, giving relative mRNA abundance of HCV, p53, p73 and RASSF1A. Huh-7.5 cells that were transfected with HCV genotype 1b or treated with pLentivirus, showed a gradual down regulation of the three tumor suppressor genes and a gradual increase in HCV genotype 1b load over the course of 16 weeks. Huh-7.5 cells that were transfected with HCV genotype 1b and then treated with c-hsa-miR-602, LNA-anti-miR-122 alone or in combination with c-hsa-miR-602 showed down regulation in the three tumor suppression genes, before selection (BS) (day 18 of transfaction with HCV). From 4 weeks onwards, the expression for the three tumor suppressor genes started to move towards normal levels, and also displayed a decrease in HCV genotype 1b load. It was also observed that Huh-7.5 cells that were transfected with HCV genotype 1b and treated with c-hsa-miR-602 alone, showed a gradual decrease in HCV load when compared to LNA-anti-miR-122 alone or in combination with c-hsa-miR-602.
In this study, we transfected the Huh-7.5 cells with HCV genotype 1b, and then treated the transfected Huh-7.5 cells with c-hsa-miR-602, LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602. We observed that c-hsa-miR-602, LNA-anti-miR-122, or LNA-anti-miR-122 in combination with c-hsa-miR-602 treatments were effective in lowering the HCV load in infected Huh-7.5 cells. However, transfected or un-transfected Huh-7.5 cells treated with LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602 showed morphological changes of the cells after 12 weeks. Transfected Huh-7.5 cells with and without HCV genotype 1b treated with c-hsa-miR-602 showed no morphological changes of the cells with increased HCV elimination. Hence, our in-vitro findings showed that LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602 showed morphological changes of the cells after 12 weeks. Transfected Huh-7.5 cells with and without HCV genotype 1b treated with c-hsa-miR-602 showed no morphological changes of the cells with increased HCV elimination. Hence, our in-vitro findings showed that LNA-anti-miR-122 or LNA-anti-miR-122 in combination with c-hsa-miR-602 showed morphological changes of the cells after 12 weeks.

Tumor suppressor genes are capable of promoting apoptosis, controlling cell division and suppressing metastasis. The loss of tumor suppressor gene function may lead to development of cancer. Transformation of normal cells to cancer cells requires genetic mutations in proto-oncogenes and/or tumor suppressor genes. These mutations can be directly induced by radiations, hormones, chemicals, genetic factors and viruses. Inactivation of a tumor suppressor gene leads to uncontrolled cell growth a defining feature of cancer. RASSF1A, p53 and p73 are tumor suppressor genes that inhibit tumor development.

We observed that Huh-7.5 cells transfected with only HCV genotype 1b and treated with pLentivirus showed a gradual down regulation of the three tumor suppressor genes: RASSF1A, p53 and p73, with a gradual increase in HCV accumulation over the course of 16 weeks. Our results showing the down regulation of the three tumor suppressor genes in HCV transfected Huh-7.5 cells was correlated with the finding of HCC cell line (N=27) model data from CCLE, also showing that most HCC cell lines showed down regulation of TP53, TP73, and RASSF1 (Figure 5A). Guo et al. [43] found that the expression of RASSF1A was lowered in the hilar cholangiocarcinoma cells transfected with HCV core (HCVc) protein than in normal biliary duct cells. Wu et al. [44] studied the changes in transcription or translation of c-myc and p53 genes by transfecting...
HCV F gene-containing plasmid pcDNA3.1-F and HCV core gene with pcDNA3.1-C in HepG(2) liver cells and showed that the levels of c-myc expression were up-regulated and those of p53 expression were down-regulated by HCV F protein. Mihar et al. [45] investigated mRNA expression of p73 in 48 untreated HCC patients. RT-PCR analysis revealed that p73 mRNA was expressed ubiquitously at low levels in all the tumor tissues, as well as in the adjacent normal liver tissues. Clinical HCC tumor tissue as well as HCC cell line data for TP53, TP73, and RASSF1 from TCGA revealed that the expression of all these three tumor suppressor genes were down regulated. These results suggest that low levels of p53, p73 and RASSF1A may play a role in hepatocellular carcinogenesis that correlates with our in vitro findings. Świątek-Kościelna et al. [46] and Liu et al. [47] demonstrated that either only ribavirin or IFN-α, or a combination of ribavirin and IFN-α resulted in greater p53 activation and HCV suppression. Similarly, we found that the expression of p53, p73 and RASSF1A tumor suppressor genes in HCV transfected Huh-7.5 cells treated with c-hsa-miR-602 approached the normal levels which occurred at a slightly faster rate for p53 with a decrease in HCV load.

In conclusion, our previous and present in vitro studies suggests that hsa-miR-602 may be a potential candidate for treating HCV genotype 1b infection by regulating p53, p73, and RASSF1A tumor suppressor gene expression.

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Conflicts of interest
The authors declare that they have no conflict of interest to the research reported.

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