Heat shock reduces HCV replication via regulation of ribosomal L22 in Alu-RNA molecule dependent manner

Hamada Farghaly, Adel A. Guirgis, Hany Khalil

Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 79, Egypt.

Correspondence to: Dr. Hany Khalil, Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 79, Egypt. E-mail: hkhalil74@gmail.com


Abstract

Aim: Hepatitis C virus (HCV) infection is a global health problem that affects more than 180 million people worldwide. HCV is associated with several hepatic and other hepatic disorders including malignancies. HCV is a small enveloped positive-single strand RNA virus that belongs to Hepacivirus in the family Flaviviridae. Here we aim to provide a new therapeutic strategy via treatment of infected HepG2 cells with heat shock (HS).

Methods: The potential inhibitory effect of HS on HCV replication was assessed by the relative gene expression of NS5A and its corresponding protein by flowcytometry which has been additionally used to monitor other cellular factors.

Results: HS treatment of infected HepG2 cells has the ability to disturb HCV replication possibly via stimulation of the Alu non-coding element which inhibits gene expression of ribosomal L22. Ribosomal protein L22 (RPL22) is one of the abundant RNA-binding proteins that are known to facilitate synthesis and translation of viral RNA and to participate in balancing the protein components of the ribosome itself.

Conclusion: HS treatment of infected cells leads to up-regulation of long RNA-Alu molecule that regulates the expression of RPL22 and subsequently reduces HCV replication in HepG2 cells.

Keywords: Hepatitis C virus, Alu non-coding gene, heat shock treatment
INTRODUCTION

Hepatitis C virus (HCV) infection is the most common cause of chronic liver disease that is considered as the common sign of liver transplantation in United States, Australia and European countries. Almost 3% of global population are infected with HCV which mean approximately 180 million people worldwide. HCV belongs to the family Flaviviridae which replicates in the cytoplasm of liver hepatocytes\(^1,2\). HCV acute infection is most often asymptomatic leading to chronic infection of about 75% patients. The manifestation of chronic HCV is directly alternated from asymptomatic state to cirrhosis and hepatocellular carcinoma (HCC) phase. Indeed, HCV infection is slowly progressed without clinical appearance in the liver of many patients. Therefore, approximately 20%-30% of infected individuals may develop liver cirrhosis over 20 to 30 years period of infection\(^3\). HCV genome encodes a large open reading frame (ORF) which have translated into polypeptide chain with approximately 3000 amino acids that have cleaved into ten proteins. Three structural proteins including the core, E1 and E2, in addition to five non structural proteins contain NS3 (helicase/protease), NS4A, NS4B, NS5A, and NS5B (RNA-dependent RNA polymerase)\(^4\). HCV entry to the host involves a complex series of interactions including attachment, fusion and entry. Attachment of HCV with host receptor and co-receptors is facilitated by heparin sulfate proteoglycans that are expressed on hepatocytes surface. Meanwhile, LDL receptor (LDLR) binds to HCV and promotes its virion entry in pH-dependent by clathrin-mediated endocytosis\(^5-7\). During entry process, many cellular factors have been identified, including the scavenger receptor class B type I (SRB1), CD81, tight junction proteins, claudin-1 (CLDN1) and occludin (OCLN)\(^8,9\). SRB1 and CD81 have been identified as binding partners of HCV that are highly expressed in the liver and increase the selective uptake of HDL cholesterol esters into hepatocytes\(^9,10\).

Interestingly, targeting of these receptors and others cellular factors provides potential avenues to prevent HCV infection and suggests that modulation of their physiological role does not lead to significant toxicity on host cells. Generally, treatment of HCV contains several drugs that directly interact with viral proteins such as symeprevir, grazoprevir and asunaprevir (NS3 inhibitors) which inhibit HCV-NS3 proteases\(^11,12\). Several drugs target HCV-NS5B polymerases (NS5B inhibitors) such as sofosbuvir and dasabuvir, while others interact as NS5A inhibitors such as daclatasvir, elbasvir and ledipasvir\(^13\).

Importantly, over-use of antiviral drugs is considered as a factor for development of viral-escape mutation, which leads to rapid HCV resistance. Recently, genome wide RNAi screening revealed many host cell factors that are essential for the replication of HCV\(^14\). These factors are attractive candidates for potential antiviral medications as it is less likely that HCV will develop resistance rapidly to drugs that target host cell factors. Several studies have been reported on the essential role of heat shock treatment and its associated proteins (HSP) during viral replication including HSP27, HSP70 and HSPB8\(^15-17\). Such stress proteins have crucial impact in viral entry, activation, life cycle and assembly of human immunodeficiency virus (HIV)\(^15\). HSPB8 showed competitive antiviral activity through direct interaction with HCV-NS4B protein\(^17\). Further, HSPs is able to prevent the inflammatory damage and promote the production of anti-inflammatory cytokines indicating the potential imunuoregulatory role of HSPs\(^16\). Interestingly, one of HS response properties is the activation of non-coding RNA-Alu repeats which interact as inhibitory elements of transcription process\(^19\). A variety of long non-coding RNAs molecules (Inc-RNAs) are transcribed in mammalian cells to post-transcriptionally regulate gene expression. Lnc-RNAs play crucial roles in modulating mRNA stability, regulating mRNA translation and mediating protein modifications. Alu non-coding element is the most abundant repetitive RNA elements in the human genome. Recently, several studies demonstrated that Alu molecules modulate gene expression at the post-transcriptional level\(^20,21\). On the other hand, ribosomal proteins (RPs) highly contain RNA-binding sites with auxiliary functions, particularly by the viruses, which are so adept at usurping the cellular machinery\(^22\). Ribonucleoproteins are responsible for synthesis of new proteins beside other critical functions including the fundamental three-dimensional structures of small and large RNA molecules in ribosomal subunits\(^24\). One of these ribosomal proteins is RPL22 which has the ability to interact and support HCV-RNA translation\(^24\). In the current work, we investigated the potential up-regulation of the long RNA molecule, Alu, in response to HS in HepG2 cells that were pre-infected with...
HCV genotype 4. The potential targeted gene by activated Alu molecule has been detected using qRT-PCR and flowcytometry. HCV replication in treated cells has been monitored to figure out the inhibitory effects of HS on viral replication.

**METHODS**

**HepG2 cell line**

HepG2 cells were obtained from VACSERA, Giza, Egypt and were propagated in order to obtain increasing numbers of cells for further investigations. Propagation was done using RPMI media which supplemented with 1% L-glutamine, 10% bovine serum albumin (BSA) and 1% penicillin/streptomycin at 37 °C at CO₂ incubator.

**HCV infection**

Blood sample from a patient with HCV genotype 4 was identified and provided from Ain Shames Specialized Hospital, Egypt. For infection, HepG2 cells were incubated for three days with the serum of derived sample in multiplicity of infection (MOI) of 0.5[^25].

**HS treatment and virus infection**

To figure out the effect of HS on HCV replication, HepG2 cells were seeded in 6-well plates (2 × 10⁵ cells per well). Cells were infected for 3 days with HCV (MOI = 0.5), other cells were incubated without infection. All cells were then stimulated by HS using warm media (45 ºC for 5 min). The infectious media was collected and stored at -80 ºC for LDH detection as an indicator for cytotoxic effect of heat shock.

**Cytotoxic effect and metabolic activity of host cells**

To determine the time cytotoxic 50% (TC₅₀) of HS, HepG2 cells were seeded in 96-well plates in a density of 5 × 10⁴ cells per well. The cells were then treated for different time point (0-10 min) with warm media (45 ºC). After each incubation period, the cytotoxic effect was monitored by using water-soluble tetrazolium salt (Cell proliferation reagent WST-1, Sigma, USA) according to the manufacture protocol. The number of living cells was calculated and cell survival was investigated by using inverted microscope and detection of lactate dehydrogenase (LDH) level using LDH detection kit (Abcam, ab102526). According to the manufacture procedures, equal amounts of infections medium and LDH buffer (40 µL) were incubated with LDH substrate for 1 h then the relative LDH production was calculated according to the standard curve. Cells that were treated with 50 and 100 µL of Triton x-100 served as a positive control[^26].

**RNA isolation and quantitative real time-PCR**

Total RNAs were isolated from treated and untreated cells using TriZol (Invitrogen, USA) and chloroform methods. Isolated RNA was dissolved in RNase free water and the concentration of all samples was adjusted to final concentration of 100 ng/µL. Then 10 µL from each isolated and purified total RNA was used to generate cDNA using cDNA synthesis kit (Qiagen, USA). According to the manufacturer protocol, total RNA was incubated with reverse transcriptase and poly (dT) primers at 45 °C for 1 h followed by 5 min incubation at 95 ºC. The cDNA was then incubated at -20 ºC until used[^27,28]. q RT-PCR was used to detect the relative expression of viral NS5A, non-coding Alu gene and L22 ribosomal gene in infected HepG2 cells upon heat shock treatment compared to control, the qRT-PCR was performed by using SYBR green and the following oligonucleotides specific for NS5A, Alu and L22 genes; NS5A-For-5’-ATTCGTTCGTAGTGGGATCCA-3’, NS5A-Rev-5’-AAGAGTCCAGTATTATCACCTT-3’, Alu-for-5’-AAAAACGGTGAAACCCCGGT-3’, Alu-rev5’-TATGTGCCAGGCACCTTTT-3’ and L22-for-5’-GAATTCGCACCGACTGCAGT-3’ and L22-rev-3’-GGTGTTTCGCAAAAGGTGCTGATCCC-5’. Levels of GAPDH, as internal control, were amplified using specific oligonucleotides GAPDH-for 5’-TGGCATTGGAAGGGCTCA-3’ and GAPDH-rev-5’-TGATGCAGGGATGTTTCT-3’. The following parameters have been used in qRT-PCR program, 94 ºC for 3 min, 40 cycles (94 ºC for 15 s, 60 ºC for 30 s and 72 ºC for 30 s) and finally 72 ºC for 10 min[^19,24].
Flow cytometry analysis
HepG2 cells were seeded in 6-well plates in concentration of $2 \times 10^5$ cells per well, 25 µL serum of infected patient with HCV genotype 4 that contains $1 \times 10^5$ virus (MOI = 0.5) was added to each well, and incubated for 3 days at CO$_2$ incubator. The old media was removed and fresh warm media (45 ºC) was added for infected cells for 5 min. Then the media has been removed and cells were washed using PBS, and trypsinized by using trypsin. Finally the cells were collected in PBS and centrifuged at room temperature at 5000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in PBS that contains Triton-X-100 (0.01%) for permeabilization, then cells were centrifuged as previously described. The supernatant was removed and the pellet was resuspended in PBS that contains 1% BSA and 1:1000 diluted rabbit monoclonal antibodies for either NS5A or RPL22 protein (Promega, USA) followed by 1 h incubation at room temperature. The cells were centrifuged as previously described and were washed using PBS for three times. The cells were then incubated for 1 h in the dark with secondary antibody goat anti-rabbit (Promega, USA) in dilution of 1:100. Finally, the stained cells were centrifuged and washed by PBS and were collected in 500 µL PBS for flowcytometry (Becton Dickinson Facscalibur).

Prediction tools
To investigate the possible interaction and potential binding site between Alu-repeated sequence and RPL22 sequences, IntaRNA software has been used.$^{[29-31]}$

Statistical analysis
A student’s two-tailed test was used to determine significance values of relative gene expression in treated and non-treated cells. SDS 2-2.2 software was used to analyze the Ct values of the q RT-PCR and to drive and calculate the relative gene expression using $\Delta\Delta$Ct equations.$^{[32]}$

RESULTS
Heat shock has no cytotoxic effect on cell viability rate
HS is the consequences for subjecting the cells to higher temperature than the optimal temperature range for biological functions. The influence of HS on cells’ viability rate was monitored dependent on cell imaging, number of living cells and TC$_{50}$ following incubation. Additionally, lactate dehydrogenase (LDH) production from treated cells was measured as an indicator for systemic toxic effect of HS. HepG2 cells were seeded in either 6-well plates or 96-well plates and were incubated overnight. Next, the cells were infected with HCV by adding the patient-derived serum to fresh media (MOI = 0.5) followed by 3 days of incubation. The infected cells were subjected to warm media (45 ºC) for the indicated time points. Cell viability rate dependent HS-time course was detected by using WST-1 assay which revealed that the TC$_{50}$ is greater than 10 min [Figure 1A]. This result indicates that the cytotoxic effect of HS is initiated upon 10 min of treatment. Furthermore, cells imaging and living cells upon 5 min of HS treatment showed no detrimental influence on treated cells in comparison with cells that were left without treatment (NT) [Figure 1B and C]. The relative LDH production showed negligible differences between 5 min-HS-treated cells, non-treated cells (NT) and mock in comparison with cells that were treated with Triton-X 100 as detergent agent [Figure 1D]. These data reveal that treatment with HS (45 ºC) for 5 min has no cytotoxic effect in HepG2 cells.

HS treatment disturbs HCV replication via regulation of viral NS5A gene
In order to investigate whether the HS treatment has an influence on HCV replication, the relative gene expression of viral NS5A and its corresponding protein level have been detected in HepG2 cells following HS treatment. NS5A is a zinc-binding and proline-rich hydrophilic nonstructural protein that plays a crucial role in HCV-RNA replication. NS5A has the ability to modify NS5B polymerase activity and modulate multiple aspects in cellular immune response.$^{[33]}$ Thus, the expression of NS5A reveals the capability of viral replication in infected cells. Here, the expression of HCV-NS5A has been detected at both RNA and protein...
levels using qRT-PCR and flowcytometry, respectively. Our results showed that the relative expression of NS5A was decreased in HepG2 cells that were pre-treated with HS in comparison with control infected cells indicated by qRT-PCR [Figure 2A]. Further, the statistical analysis of mean values calculated from cycles threshold (CTs) revealed a significant differences of NS5A expression in HS treated cells compared to control infected cells \((P > 0.05)\). Moreover, the expression of NS5A protein in HepG2 cells has been detected in 30% of total cells that were pre-treated HS. While 70% of total control infected cells revealed normal level of NS5A that indicated by flowcytometry [Figure 2B]. These results indicated that HS stress could prevent HCV replication in HepG2 cells via depletion of its NS5A expression at both RNA and protein levels.

**HS inhibits RPL22 gene expression via stimulation of Alu-RNA in HepG2**

To investigate the effect of HS on Alu-RNA elements and its potential targeted gene RPL22, the relative expression of Alu and RPL22 genes were detected in HepG2 treated cells compared to control infected cells using qRT-PCR. Our findings showed that the relative expression of Alu molecule has been significantly accumulated in response to HS treatment in HepG2 infected cells in comparison with control infected cells \((P = 0.009)\) [Figure 3A]. Meanwhile, the relative gene expression of ribosomal RPL22 was significantly reduced in infected HepG2 cells that were subjected to HS \((P = 0.001)\) [Figure 3B]. These data suggest that HS stress leads to activation and accumulation of Alu-RNA elements that may regulate the expression of ribosomal RPL22 gene in infected cells. In order to investigate the potential binding site of RPL22 by Alu-repeat sequences, IntaRNA software was used. The docking interaction indicates a seeding region on target location (11-26) by the query location (173-186) [Figure 3C]. These findings indicate the possible regulation of RPL22 messenger RNA (mRNA) by Alu elements in response to HS treatment.
L22 ribosomal protein is regulated by HS stress condition

To assess the inhibitory effect of HS on RPL22 protein, the protein profile of RPL22 has been detected in HepG2 cells that were infected with HCV and subjected to 5 min HS using flowcytometry. The result showed that approximately 25% of total HepG2 cells were positively expressed RPL22 in response to HS treatment, while 40% of total cells were positive to RPL22 in control-infected cells [Figure 4]. These findings indicate that few minutes of HS stress lead to obvious depletion of RPL22 in HepG2 cells. Together, our data demonstrate that HS treatment is an environmental stress leading to accumulation of Alu-RNA element that post-translationally regulates RPL22 and subsequently disturbs HCV replication in HepG2 cells.

DISCUSSION

Our findings provide a new therapeutic strategy against HCV infection without detectable toxic effect on cell viability. Treatment with HS (45 ºC for 5 min) is able to decease virus replication indicated by viral NS5A expression at RNA and protein levels. This interruption in viral replication may be due to up-regulation of Alu repeats element as a response to HS. Alu molecule is non-coding RNA that is present at elevated levels in stress condition. Consequently, Alu repeats are increasingly being associated with the physiological stress response[19]. Alu sequences are the most abundant short interspersed repeated elements in the human genome. The accumulation of Alu-RNA molecules has been observed in variety of cancer cells in association with cellular microRNA[33]. However, the exact molecular function of Alu-RNA element is still not completely

Figure 2. Heat shock (HS) reduces HCV-NS5A gene expression profile. (A) Relative gene expression of HCV-NS5A in HepG2 cells that were stressed with HS for 5 min in comparison with non-treated and infected cells (control-infection); (B) the expression of NS5A corresponding protein in HS treated cells (HS-infection) compared to control-infected cells indicated by Flowcytometry. Error bars indicate standard deviation of two independent experiments
Figure 3. Down-regulation of L22 in Alu-RNA element dependent manner. (A) Relative expression of Alu non-coding gene in cells that were treated with heat shock (HS) in comparison with non-treated cells (control) using qRT-PCR; (B) relative expression of L22 gene in cells that were treated with HS in comparison with non-treated cells (control) using qRT-PCR. Error bars indicate the standard deviation of two independent experiments; (C) the possible binding site and seeding region of Alu repeated sequences (query) and RPL22 sequences (target) indicated by IntaRNA software.

Figure 4. Interruption of L22 corresponding protein in response to heat shock (HS). The expression protein level of RPL22 elevated by percentage of positive HepG2 cells that were treated with HS and infected with HCV compared to control infected cells using flowcytometry.
understood. Recent studies have demonstrated that Alu RNA plays a major role in post-transcriptional regulation of gene expression for example by affecting alternative splicing, mRNA stability and protein synthesis\(^{33,34}\). One of the recent identified targets that is modulated by Alu-RNA is the RPL22-mRNA\(^{20}\). Thus, here our findings further confirm the regulatory effect of Alu-RNA molecules on gene expression of RPL22 that activated upon HS treatment. Interestingly, regulation of RPL22 in Alu-RNA dependent manner leads to significant interruption of HCV replication in HepG2 cells. Therefore, the current data provide a new technique that can prevent HCV replication in host cells without a harmful effect on treated cells as compared with non-treated cells. HS refers to cellular exposure to rapid stress changes such as temperature, toxins, oxidative stress, heavy metals, and pathogenic infections. Specifically temperature induced HS, even of a few degrees, has the ability to disturb protein folding. Other cellular damages have been reported in response to HS stress including rearrangement of cytoskeleton, alternation of organelle location, decreasing of ATP production, decreasing of proteins translation, changes in RNA splicing and gene silencing\(^{35}\). The present data indicate the possible regulation of RPL22 expression in infected HepG2 cells that were subjected to HS. RPL22 is an RNA-binding protein with 60S large ribosomal subunit that plays a crucial role of macrolide resistance in bacteria\(^{36}\). In vertebrates, RPL22 mutation might increase the proliferation of cells and then increase cancer risk. However, RPL22 has not been implicated in any lung diseases, especially in lung cancer\(^{36,37}\). Other study demonstrated that human RPL22 protein interact with HCV-NSSA and support viral RNA translation\(^{38}\). NSSA protein is the most common HCV research regarding its potential regulation of cellular immune response following infection. NSSA protein is translated from HCV genome as one of a large number of ploy-proteins that processed by NS3 protease\(^{39}\). NSSA protein modulates host interferon signaling via direct interaction with the cellular factor retinoic acid-inducible gene-I (RIG-1) protein resulted in blocking of interferon signaling in infected cells\(^{40}\). Additionally, NSSA plays the key role during HCV replication cycle and viral particles assembling through interaction with several viral and host proteins to insure viral replication. Several evidences indicate that NSSA is localized in certain modified cytoplasmic membrane during HCV replication that facilitates its significant role in HCV replication complex and replicase\(^{41,42}\). Here, the relative expression of NSSA has been detected by q-RT-PCR using newly designed specific oligonucleotides. Our results showed that NSSA relative expression was significantly reduced in infected cells that were subjected to HS in comparison with control infected cells. On the other hand, flowcytometry has been used to investigate the clearance status of NSSA protein in HepG2 cells that were treated with HS. Interestingly, in comparison with control infected cells, our findings reveal that the percentage of NSSA positive cells was 30% of infected cells that were treated HS. Meanwhile, the percentage of NSSA positive cells was up to 70% regarding the control infected cells. These data demonstrate that HCV replication is potentially interrupted in HepG2 cells that were subjected to 5 min of HS. Taken together, these data provide an evidence for the possible inhibition of HCV infection via HS treatment affecting the expression of RPL22 through activation of Alu non-coding repeated element in HepG2 cells.

DECLARATIONS

Acknowledgments

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Authors’ contributions

Planned and designed the study: Khalil H
Did the experiments assessing and developments: Farghaly H, Guirgis AA, Khalil H
Wrote the manuscript: Khalil H

Availability of data and materials

The data and materials are obtained from the corresponding author.
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Conflicts of interest
All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate
This study was approved by the ethical committee for post graduate studies of the University of Sadat City.

Consent for publication
Not applicable.

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