Predictive prognostic role of *miR-181a* with discrepancy in the liver and serum of genotype 4 hepatitis C virus patients

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Abstract. microRNA (miRNA) expression in organs does not always represent their quantity in serum. A disparity in the expression of *miR-181a* has been reported in the tissues and serum of hepatocellular carcinoma (HCC) patients. Since hepatitis C virus (HCV) is a major cause of HCC and *miR-181a* has never been studied in HCV, the present study aimed to investigate the *miR-181a* expression profile in genotype 4 (GT4)-HCV patients to evaluate whether this pattern is also apparent in HCV. RNA was extracted from liver tissues, peripheral mononuclear cells (PBMCs) and serum samples from GT4-HCV-infected patients and healthy donors to evaluate the relative *miR-181a* expression using quantitative reverse transcription-polymerase chain reaction. *miR-181a* was significantly higher in the serum of naïve patients compared to controls, and an inverse correlation with the viral load and liver enzymes was apparent. By contrast, no difference in *miR-181a* expression was observed in the liver tissues and PBMCs of patients compared to controls. This expression observed in HCV is conflicting to that previously reported in HCC. The study also demonstrates a significant upregulation of *miR-181a* post-interferon/ribavirin treatment in the serum of sustained virological responders (SVRs) compared to non-responders and treatment-naïve SVRs. In conclusion, *miR-181a* may be considered to be a possible prognostic marker in GT4-HCV infection.

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Introduction

Several studies have investigated the source of circulating microRNAs (miRNAs); however, it is an issue of debate. Previously, it has been indicated that miRNAs are present in the circulation as a result of the release of exosomes from cells (1-3). A study by Esquela-Kerscher and Slack (4) proposed that miRNAs enter the circulation during angiogenesis following their release from tumor cells or as a result of tumor cell death. In addition, another study postulated that miRNAs may also be abundant in the circulation as a result of their release from inflamed organs (5). Changes in circulating miRNA levels do not only result from changes within tumors, but may also be a result of inflammatory reactions or host immune response. Based on this fact, the expression of miRNAs in organs does not always represent their quantity in serum. For example, *miR-195* was found to be downregulated in breast cancer tissues (6), whereas its circulating levels were found to be upregulated in breast cancer patients compared to controls (7). In support of this evidence, Waters *et al* (8) showed that *miR-195* and *miR-497* were decreased and *miR-221* was increased in tumor tissues from murine models of breast cancer compared to healthy tissues, with no difference in the expression of all three miRNAs in the circulation. Additionally, *miR-122* was shown to have a low expression in liver (9,10) and high expression in the serum of hepatocellular carcinoma (HCC) patients compared to the controls (11). *miR-181a* represents another example for this discrepancy, as upregulation of *miR-181a* was reported in embryonic and epithelial cell adhesion molecule/α-fetoprotein ($EpCAM^+/AFP^+$) liver tissues compared to the healthy controls (12), whereas at the serum level no difference in *miR-181a* expression was observed (13).

miR-181a is an immunoregulatory miRNA (14) that is reported to have its highest relative expression in the thymus, the primary lymphoid organ and site of T lymphocyte maturation (15), highlighting its role in the maturation, sensitivity and selection of T lymphocytes. *miR-181a* has shown aberrant expression in viral infections, in which it was reported to be downregulated in human papillomavirus-positive cell lines

and upregulated in HepG2 cell lines infected with hepatitis B virus (HBV) (16,17). In hepatitis C virus (HCV), a single study was conducted by Liu *et al* (18) in which a microarray was performed, which showed that infection of human hepatoma cell lines (Huh 7.5.1) with JFH-1 HCV leads to *miR-181a* downregulation.

From the aforementioned data, there is clear evidence that *miR-181a* shows aberrant expression in liver tissues that was not mirrored in the serum of HCC patients (8,12,13). Since infection with HCV is a major cause of HCC, the present study aimed to reveal the expression profile of *miR-181a* in HCV infection and correlate it to the expression reported in HCC. Therefore, *miR-181a* was screened in serum, liver tissues and peripheral mononuclear cells (PBMCs) of patients infected with genotype 4 (GT4)-HCV, the most prevalent HCV genotype in Egypt, which to the best of our knowledge has never been previously investigated. The study also aimed to associate the serum *miR-181a* expression with response to the standard therapy used in Egypt, pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy.

Patients and methods

Study subjects. A total of 72 patients chronically infected with HCV and 22 age-matched controls were included in the study. The patients were classified as 24 naïve patients, 11 sustained virological responders (SVRs) pre-treatment, 15 SVRs post-treatment, 12 non-responders (NRs) pre-treatment and 10 NRs post-treatment. All the naïve patients were candidates for PEG-IFN/RBV therapy. The presence of HCV RNA and anti-HCV antibodies in the serum was used to diagnose HCV infection. The patients were determined as negative for the hepatitis B surface antigen. The samples from post-treatment SVRs and NRs were obtained following treatment with weekly injections of PEG-IFN-α and daily oral doses of RBV at Al Kasr Al Ainy School of Medicine, Cairo University (Cairo, Egypt). The controls were healthy volunteers that were all negative for HCV, HBV and HIV infection. The patients and healthy volunteers included in the study provided their written informed consent. All the clinical procedures were performed in compliance with the guidelines of the Institutional Review Board of Al Kasr Al Ainy School of Medicine in Cairo University and in accordance with the ethical standards of the Declaration of Helsinki.

Collection of samples. Peripheral venous blood (8 ml) was collected from each patient and control in the presence of an anticoagulant, EDTA, for isolation of PBMCs. All the samples were processed on the same day and within a few hours after collection. In addition, 2 ml blood samples were collected from patients and healthy controls for serum separation. The liver tissue samples were collected from patients by fine-needle aspiration and healthy liver tissues were obtained during liver transplantation. The samples were directly cryopreserved following biopsy collection until required for use.

Serum separation and isolation of PBMCs. The serum samples were collected in serum separator tubes, centrifuged at 1,500 x g for 10 min and immediately frozen at -80˚C until required for use.

PBMCs were isolated using the Ficoll (Axis-Shield PoC AS, Kjelsåsveien, Oslo, Norway) density gradient centrifugation method as previously described (19), and frozen at -80˚C until required for use. Cell counting and viability testing were performed using trypan blue.

Genotyping. Genotyping was performed using the Versant® HCV Genotype 2.0 assay (LiPA; Bayer HealthCare, Tarrytown, NY, USA), at the National Cancer Institute, according to the manufacturer's instructions.

Cell culture. Huh7 cells were maintained in Dulbecco's Modified Eagle's medium supplemented with L-glutamine, penicillin/streptomycin and fetal bovine serum.

In vitro transcription and transfection of GT4-HCV full length genome. The intergenotypic recombinant $pED43_{5'UTR-NS2}/JFH1_{T8271,T977S}$ encompassing the 5' untranslated region (UTR) to NS2 region of the GT4a-HCV genome (provided by Professor Jens Bukh) (20) was linerized using the *Xba*1 restriction enzyme (Thermo Scientific, Waltham, MA, USA) and purified using phenol-chloroform. Confirmation of linearization was performed using gel electrophoresis. Linearized plasmid was *in vitro* transcribed using the MEGAscript T7 *in vitro* transcription kit (Ambion, Life Technologies, Carlsbad, CA, USA). The transcribed viral RNA was transfected into the Huh7 cells by lipofection (Superfect; Qiagen, Hilden, Germany). The supernatant containing HCV particles was collected and filtered through 0.45-μm pore size syringe filters for further use.

Infection. Naïve Huh7 cells were inoculated overnight with the filtered supernatant harvested from the HCV RNA-transfected cells.

Total RNA extraction and reverse transcription. Total cellular RNA was extracted from the Huh7 cells, liver biopsies, PBMCs and serum samples under sterile conditions using Biozol (Bioer Technology Co., Ltd., Binjiang Hanchuan, China) according to the manufacturer's instructions, as first demonstrated by Chomczynski and Sacchi (21). The TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was subsequently used to reverse transcribe the total extracted RNA into single-stranded complementary DNA according to the manufacturer's instructions.

Quantification of miRNAs expression. The relative expression of *miR-181a* (TaqMan miRNA; ID: 000480) was quantified using StepOne Real-Time PCR (Applied Biosystems) using the TaqMan MicroRNA assay (Applied Biosystems). The comparative cycle threshold (CT) method, which involves comparing the CT values of the samples of interest to that of the healthy controls, was used to calculate the amount of *miR-181a*. In the Huh7 cell lines, liver biopsies and PBMCs, the amount of miRNA was calculated relative to the amount of the reference gene, *RNU6B*, in the same sample. Reactions, including the controls, were run in duplicates.

Statistical analysis. miRNA expression is represented as relative quantification (RQ). For the Huh7 cells, liver tissues and

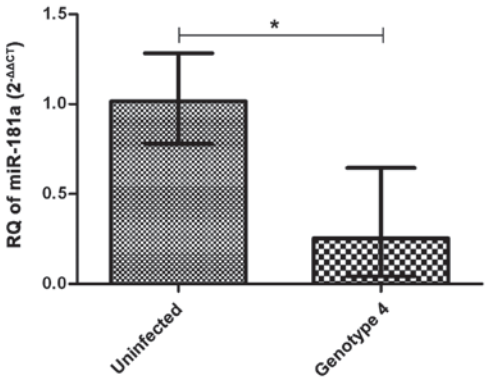

Figure 1. *miR-181a* expression pattern in HCV-infected Huh7 cells. *miR-181a* expression in GT4-HCV-infected and uninfected human hepatoma cells (Huh7) was quantified using qRT-PCR. *miR-181a* was downregulated in GT4-HCV-infected Huh7 cells compared to uninfected cells on day 1 postinfection (n=4) (*P=0.0286). Mann-Whitney U test was used for comparison and results are expressed as median with range. *miR-181a*, microRNA-181a; HCV, hepatitis C virus; GT4, genotype 4; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RQ, relative quantification.

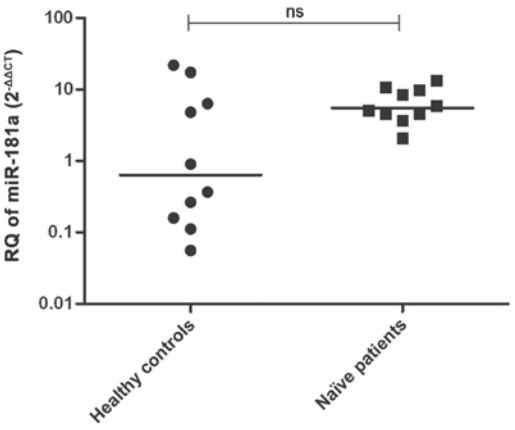

Figure 2. *miR-181a* expression pattern in liver tissues of HCV-infected patients and healthy controls. *miR-181a* expression was measured in HCV-infected (n=10) and healthy (n=10) liver tissues using qRT-PCR. No significant difference was found between the expression of the miRNA in the groups (P=0.1431). Mann-Whitney U test was used for comparison and results are expressed as median. The bar represents the median value. *miR-181a*, microRNA-181a; HCV, hepatitis C virus; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RQ, relative quantification; ns, not significant.

PBMCs samples, RQ=$2^{-\Delta\Delta CT}$, whereas for the serum samples, RQ=$2^{-\Delta CT}$, as described previously (22). Mann-Whitney U test was employed to compare miRNA expression and the results are expressed as median. Correlation studies were analyzed using Spearman test. P<0.05 was considered to indicate a statistically significant difference. Analysis and calculations were performed using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA).

Results

Expression pattern of miR-181a in HCV-infected Huh7 cells. Huh7 cells infected with pED43$_{5'UTR-NS2}$/JFH1$_{T8271,T977S}$ encompassing the GT4a-HCV genome for 24 h showed lower *miR-181a* expression when compared to uninfected cells (n=4) (P=0.0286) (Fig. 1).

Expression pattern of miR-181a in liver tissues of naïve HCV-infected patients and healthy control subjects. All the samples were found to be infected with GT4-HCV. The *miR-181a* expression in HCV-infected (n=10) and healthy (n=10) liver tissues was examined using quantitative reverse transcription-polymerase chain reaction. *miR-181a* showed similar expression in liver tissues of patients and healthy controls (P=0.1431) (Fig. 2).

Expression pattern of miR-181a in PBMCs of naïve HCV-infected patients and healthy control subjects. *miR-181a* expression was measured in the PBMCs isolated from naïve GT4-HCV-infected patients (n=14) and healthy controls (n=12). No significant difference was observed in *miR-181a* expression in patients compared to the controls (P=0.4714) (Fig. 3).

Expression pattern of miR-181a in serum of naïve HCV-infected patients compared to healthy control subjects. The *miR-181a* expression was measured in the serum samples of naïve GT4-HCV-infected patients (n=14) and was compared to the expression in the healthy controls (n=12).

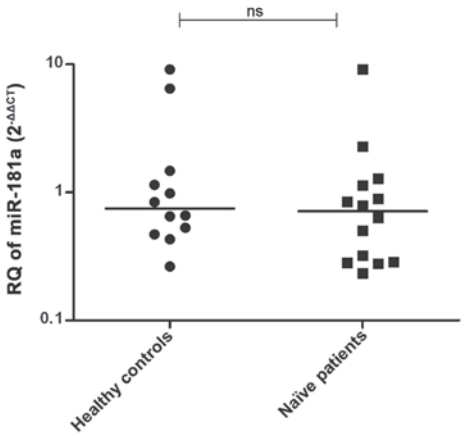

Figure 3. *miR-181a* expression pattern in PBMCs of naïve GT4-HCV-infected patients and healthy controls. Quantification of *miR-181a* expression in naïve HCV-infected patients (n=14) and healthy controls (n=12) was performed using qRT-PCR. *miR-181a* expression did not show significant differences in the PBMCs of patients compared to controls (P=0.4714). Mann-Whitney U test was used for comparison and results are expressed as median. The bar represents the median value. *miR-181a*, microRNA-181a; PBMCs, peripheral blood mononuclear cells; GT4, genotype 4; HCV, hepatitis C virus; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RQ, relative quantification; ns, not significant.

miR-181a was found to be significantly upregulated in HCV-infected patients compared to the controls (P=0.0069) (Fig. 4).

Correlation between miR-181a and clinical parameters. The *miR-181a* expression measured in the serum samples of naïve GT4-HCV-infected patients (n=14) was correlated with viral load and liver transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)]. A significant negative correlation was observed between *miR-181a* expression and viral load (P=0.0304) (r=-0.5780), ALT (P=0.0162) (r=-0.6278), as well as AST (P=0.0314) (r=-0.5752) (Fig. 5).

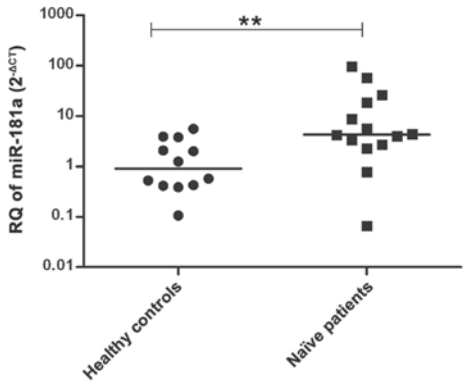

Figure 4. *miR-181a* expression pattern in the serum of naïve GT4-HCV-infected patients compared to healthy control subjects. *miR-181a* expression was quantified in naïve GT4-HCV-infected patients (n=14) and healthy controls (n=12). *miR-181a* was found to be significantly upregulated in patients compared to controls (**P=0.0069). Mann-Whitney U test was used for comparison and results are expressed as median. The bar represents the median value. *miR-181a*, microRNA-181a; GT4-HCV, genotype 4-hepatitis C virus; RQ, relative quantification.

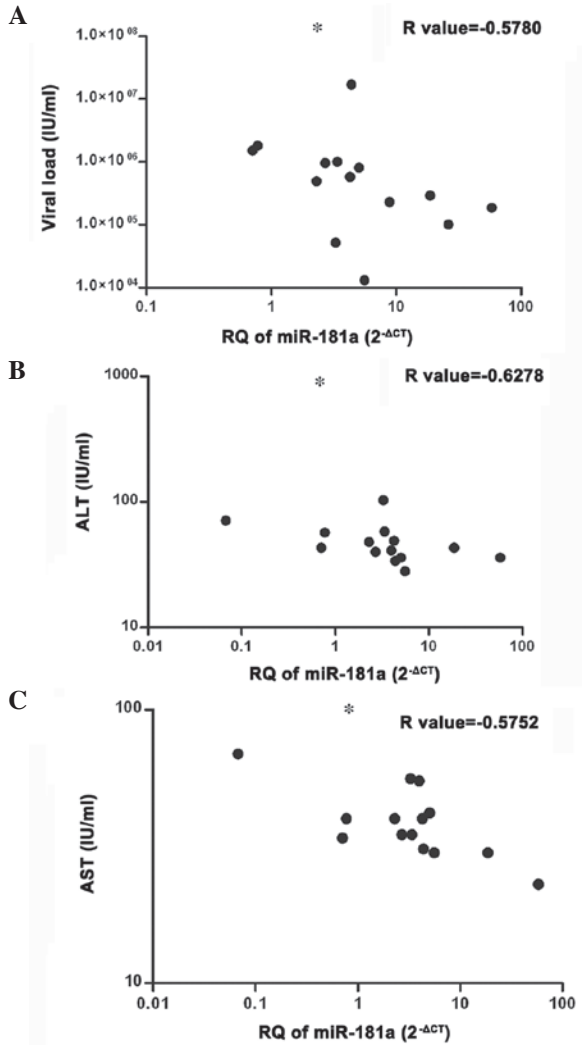

Figure 5. Correlation analysis between *miR-181a* and clinical parameters. The serum *miR-181a* expression (n=14) was correlated with (A) viral load, (B) ALT and (C) AST. The serum *miR-181a* expression exhibited a negative correlation with viral load (*P=0.0304) (r=-0.5780), ALT (*P=0.0162) (r=-0.6278) and AST (*P=0.0314) (r=-0.5752). Spearman test was used for analysis. *miR-181a*, microRNA-181a; ALT, alanine transaminase; AST, aspartate transaminase; RQ, relative quantification.

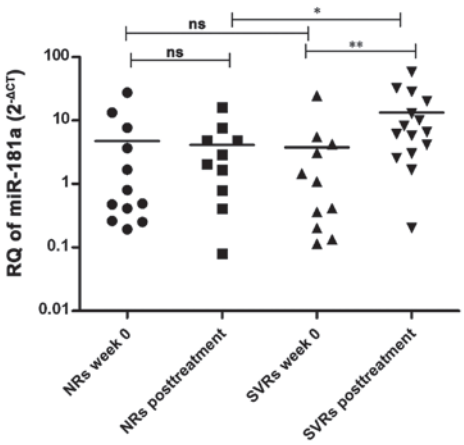

Figure 6. Variation in *miR-181a* expression in the serum of different groups of responders prior and subsequent to IFN therapy. No significant difference was found between pre-treatment levels of *miR-181a* in NRs compared to post-treatment NRs (n=12 and 10, respectively) (P=0.5095) or pre-treatment SVRs (n=11) (P=0.6891), whereas significant elevation in the expression of *miR-181a* following treatment was shown in the serum of SVRs (n=15) compared to NR patients (*P=0.0375) and pre-treatment levels in SVRs (**P=0.0075). Mann-Whitney U test was used for comparison and results are expressed as median. The bar represents the median value. *miR-181a*, microRNA-181a; NRs, non-responders; SVRs, sustained virological responders; RQ, relative quantification; ns, not significant.

Variation in miR-181a expression in the serum of HCV-infected patients of different response prior and subsequent to IFN therapy. *miR-181a* expression was assessed in the serum of SVR and NR HCV-infected patients prior (n=11 and 12, respectively) and subsequent to therapy (n=15 and 10, respectively).

No significant difference was observed in the pre-treatment expression of *miR-181a* in the serum of NR patients compared to post-treatment levels in NRs (P=0.5095) and pre-treatment levels in SVRs (P=0.6891). However, SVR patients showed higher post-treatment levels of *miR-181a* compared to pre-treatment levels in SVRs (P=0.0075) and post-treatment levels in NRs (P=0.0375) (Fig. 6).

Discussion

miR-181a was found to be highly expressed in HCC embryonic livers and $EpCAM^+/AFP^+$ HCC cells isolated from fetal livers compared to adult livers or freshly isolated mature hepatocytes (12), however, its level was similar in the serum of HCC patients and healthy controls (13). Thus far, it is not known whether *miR-181a* expression in HCV infection, as a major cause of HCC, shows the same discrepancy as reported in HCC patients. Therefore, the present study was interested in investigating the expression of *miR-181a* in GT4-HCV infection with the aim to examine whether it depicts a correlation to its profile in HCC and to the treatment response with PEG-IFN/RBV therapy, the standard therapy used in Egypt.

In order to examine *miR-181a* expression in GT4-HCV, Huh7 cells were infected with HCVcc (infectious cell culture HCV model) derived from ED43/JFH1 (provided by Professor Jens Buch). The expression of *miR-181a* was decreased in the infected Huh7 cells (Fig. 1). This finding exhibits a similarity with a study performed by Liu *et al* (18),

which showed that *miR-181a* is downregulated in Huh7 cells infected with GT2-HCV (JFH1-HCV). The finding of this study, as well as the present study, confers that *miR-181a* is downregulated in HCV-infected cell lines irrespective of the genotype. To the best of our knowledge, for the first time the expression pattern of *miR-181a* in liver biopsies, PBMCs and serum of naïve GT4-HCV-infected patients was investigated. *miR-181a* expression did not demonstrate variation in liver tissues and PBMCs of the patients compared to the controls (Figs. 2 and 3, respectively). By contrast, there was a significant increase in the serum of patients compared to the controls (Fig. 4). This elevated expression of *miR-181a* could be a result of released exosomes in the circulation (1-3). Notably, the *miR-181a* expression pattern in the liver tissues and serum of GT4-HCV-infected patients showed an inverse correlation to its expression pattern in HCC, in which HCC *miR-181a* was found to be upregulated in liver tissues and normally expressed in the serum of patients (12,13), in contrast to no expression variation in the liver tissues and a significantly increased expression in the serum of HCV-infected patients. Subsequently, whether the expression of *miR-181a* correlates to the patient clinical parameters [viral load and liver enzymes (ALT and AST)] was examined. A clear finding that serum *miR-181a* expression of HCV patients is inversely correlated with the level of viremia, as well as liver enzymes (ALT, AST) (Fig. 5), was found. To compare the *miR-181a* expression pattern among different groups of responders to standard PEG-IFN/RBV treatment, *miR-181a* was quantified in the serum of pre- and post-treatment SVRs and NRs. Serum pre- and post-treatment expression of *miR-181a* did not differ in NRs (Fig. 6). Notably, a significant upregulation of *miR-181a* was found in SVR patients following treatment compared to NR patients and treatment-naïve SVRs, with no difference shown between the groups (SVRs and NRs) prior to therapy (Fig. 6). This is in accordance with a previous study reporting comparable pre-treatment levels of *miR-122* in SVRs and NR patients infected with GT1-HCV (23). The expression of *miR-181a* observed in pre-treatment SVRs and NRs opposes that of several miRNAs depicted to show higher levels in responders compared to in NRs. For example, serum pre-treatment levels of *miR-122*, the most extensively studied miRNA in HCV, were found to be significantly higher in SVR compared to NR GT2-HCV-infected patients (24). Similarly, pre-treatment levels of *miR-122* were reported to be higher in liver tissues of responders compared to NR GT1, 2, 3 and 4 HCV-infected patients (25). Furthermore, GT1, 2 and 3 SVR patients showed high pre-treatment levels of *miR-155* in liver tissues and PBMCs, which decreased following viral clearance (26,27).

In conclusion, to the best of our knowledge, the present study demonstrates for the first time, a disparity in the expression of *miR-181a* in the liver tissues and serum of GT4-HCV-infected patients compared to controls, which is conflicting to the expression pattern of *miR-181a* reported in HCC (12,13). Additionally, although pre-treatment *miR-181a* expression did not differentiate between SVRs and NRs, the serum of SVR patients post-treatment was shown to exhibit a significant upregulation of *miR-181a* compared to NR patients and treatment-naïve SVRs, which indicates viral eradication. Thus, the data show that the upregulation of *miR-181a* in the serum of HCV patients is an indication of good prognosis and any decrease during follow-up may be an early marker for progression to HCC.

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