

DISCUSSION

Hepatitis C virus infection is one of the main causes of chronic liver disease worldwide.⁽²⁾ Persistent infection with HCV is associated with the development of chronic hepatitis characterized by chronic inflammation, steatosis and development of fibrosis leading to cirrhosis, and an increased risk of HCC.^(1,66) A number of cellular factors, demographic, and clinical characteristics, as well as viral factors, have been associated with the development of HCV-related liver damage.^(66,95) The inflammatory and fibrosis processes in HCV infection involve the induction of several hundreds of genes, a process that must be finely regulated to achieve virus clearance and to prevent the pathological consequences of a deregulated gene expression.⁽³³⁴⁾ A better understanding of the molecular mechanisms underlying the pathogenesis of HCV infection is of paramount importance for developing new therapeutic strategies.

MicroRNAs are a family of small non-coding RNAs, approximately 21-24 nt in length, that have become known as master regulators of gene expression.^(121,152) They are derived from the metabolic processing of long RNA transcripts encoded by *miR* genes in the nucleus into a shorter pre-miR, which is then exported to the cytoplasm where it is matured into small miRs.⁽¹⁴⁶⁾ The miRs' main function is to inhibit protein synthesis by protein-coding genes at the post-transcriptional level through binding to 3'-UTR coding sequences or 5'-UTR of target mRNAs, leading to mRNA degradation and/or translational repression and subsequently, gene silencing.^(152,153,156) It is estimated that more than 30% of the human protein-coding genome is regulated by miRs.⁽¹⁵⁷⁾ Individual miRs may regulate several hundred genes and vice versa, that a single mRNA may be targeted by many different miRs, giving rise to intricate regulatory networks.⁽¹⁵⁶⁾ MiRs are now recognized as major players in almost every biological process such as cell development, proliferation, apoptosis, differentiation and metabolism.⁽¹⁶³⁾ Recently, it has been shown that miRs are present not only in tissues but also can be secreted from the cells and can gain access to body fluids (e.g. plasma, serum, urine, saliva and sputum). Thus, circulating miRs can be used as non-invasive, sensitive biomarkers for detecting disease.^(181,186) To date, over 1500 miRs have been identified in the human genome so far.⁽¹⁴⁸⁾ Although most miRs are encoded individually as a single mature miR, many miRs are situated in "polycistronic" miR clusters, wherein multiple miRs are generated from the same primary transcript and are co-transcribed into a single precursor.⁽¹⁴⁹⁾

The polycistronic miR-17~92 cluster is one of the earliest characterized miR clusters. The cluster is comprised of six miRs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1).^(191,193) The miR-17~92 cluster is strongly expressed in a wide array of tissues and the sequences of the mature miRs are highly conserved in all vertebrates.⁽¹⁹⁴⁾ The *miR-17~92 cluster host gene* is located in the third intron of *C13orf25* and is shown to be the actual effector of the 13q31-q32 amplification. The cluster gene is also known as the *miR-17 host gene (MIR17HG)* (formerly *C13orf25*, *MIRH1* and *MIRHG1*).^(193,195) Although miRs are non-protein-coding, the primary transcript of *MIR17HG* contains also an open reading frame that encodes a polypeptide of 70 amino acids.⁽¹⁸⁹⁾ This polypeptide is produced by alternative splicing and has been designated as the "*MIR17HG* protein".⁽¹⁹⁵⁾ The miR-17~92 cluster has initially been linked to the initiation and progression of tumor development.^(206,219) Meanwhile, recent functional dissection of the miR-17~92 cluster indicates that individual cluster components perform pleiotropic biological functions,

which collectively regulate multiple related cellular processes such as cell proliferation, cell survival, apoptosis, cell cycle progression and the regulation of hematopoiesis and immune responses as well as organ development.^(219,222)

A number of miR-17~92 cluster targets has been identified, each of which is proposed to contribute to a specific functional readout of the miR-17~92 cluster.⁽²⁰⁶⁾ PTEN is one of the putative target genes of the miR-17~92 cluster mainly its miR-19a.⁽²⁰⁷⁾ PTEN acts as an endogenous negative regulator of the PI3K/Akt pathway, resulting in the suppression of Akt-mediated signal pathways. Both PTEN and PI3K orchestrate cell responses to growth factors, cytokines, integrins and other intercellular mediators and contribute to the growth, motility, survival and metabolic responses.⁽²⁸⁷⁾

Therefore, the present work was designed to evaluate the plasma levels of *MIR17HG* protein, encoded by the *miR-17~92 cluster host gene*, and the hepatic expression of its target PTEN in patients with chronic HCV infection in relation to hepatic steatosis, inflammation and fibrosis.

Plasma *MIR17HG* protein levels in chronic HCV infection:

The present study showed a significant increase in the plasma levels of *MIR17HG* protein in HCV-infected patients whether those with CHC or cirrhosis, compared with healthy subjects unrelated to HCV viral load. This finding may suggest that the miR-17~92 cluster was activated in chronic HCV infection. This is the first study to use the plasma *MIR17HG* protein levels as an index of miR-17~92 cluster activity. Previous studies investigating the deregulation of the miR-17~92 cluster in disease states including HCV infection, measured the levels of individual miR components of the cluster by RT-PCR. Oksuz et al (2014)⁽³³⁵⁾ reported that among the 58 serum miRs studied by quantitative RT-PCR, the expression profile of miR-17-5p, a component of the miR-17~92 cluster, was found significantly increased in HCV-positive cirrhosis group compared with control group. A recent study also showed that miR-17-5p expression in cirrhotic tissues from HCV antibody-positive patients was significantly higher than in healthy livers.⁽³³⁶⁾ Moreover, Shrivastava et al (2013)⁽³³⁷⁾ demonstrated that serum levels of the miR-17~92 cluster members, miR-20a and miR-92a, increased in patients with acute and chronic HCV infection than in sera of healthy volunteers and patients with non-HCV-related liver disease while they declined in patients with resolved infection unrelated to HCV viral loads. Also, the miR-20a and miR-92a were highly up-regulated in HCV-infected culture supernatants in comparison to that of mock-infected control.⁽³³⁷⁾ During *in vitro* acute HCV infection, 108 miRs were identified whose expression levels changed for more than 2.0-fold in response to HCV infection including up-regulation of miR-92b and down-regulation of miR-18a in human hepatoma cells using a comprehensive microarray analysis.⁽³³⁹⁾ In addition, Tsubota et al (2014)⁽³⁴⁰⁾ found that the expression levels of miR-17-5p in liver tissues of untreated patients with HCV genotype 1b were significantly associated with a poor virologic response after pegylated IFN- α and ribavirin combination therapy. Another study showed that the expression level of miR-18a in sustained virological responders was significantly lower than that in the non-responders.⁽³⁴¹⁾ Furthermore, previous studies showed up-regulation of c-Myc, the upstream of the miR-17~92 cluster, in the livers of HCV-infected patients,⁽³⁴²⁻³⁴⁴⁾ in a transgenic murine model expressing the entire HCV open reading frame and in hepatocyte cell lines harboring an HCV replicon and the infectious HCV strain JFH1.⁽³⁴²⁾ Mechanistically, activation of Akt

by the HCV NS5A, and the subsequent stabilization of the transcription factor β -catenin, was demonstrated to be responsible for activation of the c-Myc promoter, and for increased c-Myc transcription.⁽³⁴²⁾ Also, the HCV core enhances the activation of the transcription factor E2F-1, an inducer of the miR-17~92 cluster, in human liver cells by decreasing in the pRB levels.^(345,346)

Recently, it has been shown that miRs are present not only in tissues but also can be secreted from the cells into body fluids (e.g. plasma and serum) and resist degradation by endogenous ribonucleases through their incorporation into different types of vesicles.⁽¹⁸¹⁾ Thus, circulating miRs have been evolved as possible non-invasive, sensitive biomarkers for detecting disease.^(185,186) In the present study, the detection of the *MIR17HG* protein in plasma of patients with chronic HCV infection may indicate that the *MIR17HG* protein, possibly secreted by hepatocytes, could gain access to the circulation like miR components of the miR-17~92 cluster and thus, could serve as a simple, non-invasive blood biomarker for detecting HCV-related liver disease using ELISA. The sensitivity and specificity of the plasma *MIR17HG* protein levels in discriminating patients with chronic HCV infection from healthy subjects were 83.3% and 100% respectively at a cut-off level of 26.6 pg/ml in the present study. Li et al (2010)⁽³³⁸⁾ also showed that the miR-92a, a component of the miR-17~92 cluster, could separate the control and HCV groups with a high sensitivity of 97.9% and specificity of 99.4%.⁽³³⁸⁾

Like HCV, the miR-17~92 polycistron is also up-regulated in HBV infection. Inducing HBV replication in a human hepatoma cell line increased miR-17-5p, miR-20a and miR-92a-1 expression, which was reversed by c-Myc knockdown.⁽³⁴⁷⁾ Moreover, Li et al (2010)⁽³³⁸⁾ found that the serum miR-92a expression level in HBV patients was 13.6 ± 3.5 -fold of its level in healthy controls detected by quantitative RT-PCR assay and could also help to distinguish HBV infection from HCV infection. Also, differential expression of miRs was compared between liver tissues of HCV- and HBV-related liver disease and between Huh7.5 cells transfected with HBV and HCV clones and showed that miR-20 exhibited higher expression levels in the HBV group than in the HCV group. It has been suggested that endogenous miRs may be consumed and reduced by defense mechanisms, especially those against RNA viruses like HCV.⁽³⁴⁸⁾ Also, other virus infections can deregulate the expression of the miR-17~92 cluster. Retroviruses integrate nearby or within the gene that contains the miR-17~92 polycistron and induce the expression of the cluster.⁽³⁴⁹⁾ Human papillomavirus transactivates the expression of the miR-17~92 family on chromosome 13 by inducing the expression of c-Myc and through degradation of pRB, which frees E2F from the pRB–E2F complex.⁽³⁵⁰⁾ In addition, it has been shown that Epstein–Barr virus miRs and the miR-17~92 cluster could target distinct sites in the same mRNAs to exert either additive or synergistic down-regulation.⁽³⁵¹⁾

It has been reported that human miRs may be involved in defense mechanisms, mainly against RNA viruses. The interaction between miRs and viral RNAs inevitably leads to repression of viral RNA function and inhibition of viral replication.^(352,353) Moreover, the miR-17~92 cluster has a role in the anti-viral immune response. Specifically, the miR-17~92 cluster promotes T-cell proliferation, protects T cells from antigen-induced cell death, enhances Th1 cell responses and supports IFN- γ production.^(229,233,234) Moreover, the miR-17~92 cluster enhances rapid expansion of CD8⁺ T cells during the acute anti-viral responses by promoting cell-cycle progression and mTOR signaling in effector CD8⁺ cells.⁽²³⁵⁾ However, viruses may evolve strategies to

avoid miR targeting or to evade the immune clearance for optimal fitness within the host. It has been found that viruses encode also miRs that target specific host genes and pathways to prolong the longevity of infected cells or escape immune surveillance, which may be beneficial for viral infectivity and/or proliferation.⁽³⁵⁴⁾ Moreover, a significant portion of viral miRs mimics host miRs with similar target sites.⁽³⁵⁵⁾ Importantly, it has been speculated that viral RNAs may act as sponges and can sequester endogenous miRs within infected cells, thus cross-regulating the stability and translational efficiency of host mRNAs with shared miR response elements. This crosstalk between viral RNA-miR-host mRNA is termed “competitive viral and host RNAs” (cvhRNAs)⁽³⁵⁶⁾ and is dependent on the relative abundance of virus vs. host RNAs, levels of common miRs, and the number of miR response elements.⁽³⁵⁷⁾ Recent experimental evidence for the existence of cvhRNAs networks in HBV, and possibly in HCV has been reported.^(358,359) The cross-talk and reciprocal interactions between HBV/HCV RNAs and host miR-mRNAs may play an important role in regulation of host immune responses in the liver microenvironment that induce immune tolerance towards viruses and contribute to T cell tolerance and exhaustion by long-term intensive viral antigenic stimulation resulting in persistent viral expression and replication.⁽³⁵⁶⁾

PTEN as a direct target of the miR-17~92 cluster in chronic HCV infection:

PTEN is one of the main direct targets of the miR-17~92 cluster mainly its miR-19a.⁽²⁰⁷⁾ Like miRs, the miR-17~92 cluster mediates PTEN inactivation at post-transcriptional level through direct binding to 3'-UTR coding sequences of PTEN mRNA, leading to its degradation and/or translational repression and subsequently, gene silencing.^(153,208) In the present study, the plasma levels of *MIR17HG* protein were inversely correlated with intrahepatic PTEN expression in patients with chronic HCV infection suggesting that activation of the miR-17~92 cluster was associated with down-regulation of PTEN in HCV-infected livers. Similarly, expression analyses revealed that the PTEN transcript was inversely correlated with miR-17~92 expression levels in cardiac muscle tissues.⁽³⁶⁰⁾ Moreover, PTEN was up-regulated after the miR-17~92 cluster silencing in pediatric high-grade glioma cells suggesting that PTEN down-regulation could result from the overexpression of the miR-17~92 cluster.⁽³⁶¹⁾ Also, transfection of inhibitors of miR-17~92 was associated with increased mRNA and/or protein expression of PTEN in glioblastoma specimens.⁽³⁶²⁾ In addition, diet-derived butyrate, a histone deacetylase inhibitor, decreased expression of miR-17-92 cluster miRs with a corresponding increase in miR-17-92 target genes, including PTEN while introduction of miR-17~92 cluster miR mimics reversed this effect and decreased levels of PTEN in colorectal cancer cells.⁽³⁶³⁾ In the meantime, the miR-17~92 cluster may inhibit PTEN through NF- κ B activation. It has been shown that NF- κ B could down-regulate PTEN at the transcriptional levels by inhibiting its promoter transcription activity directly.⁽³⁶⁴⁾ The present study showed that PTEN expression was inversely correlated with NF- κ B expression in HCV-infected livers.

Down-regulation of PTEN has been reported in HCV-infected livers and HCV expressing cells.^(315,324,365-368) Moreover, a previous study revealed the presence of phosphorylated PTEN in HCV-infected cells.⁽³¹⁵⁾ Phospho-PTEN is an inactive form of PTEN phosphatase, which favors the activation of PI3K/Akt pathway.^(368,369) Also, Bao et al (2014)⁽³⁶⁷⁾ found marked inhibition of nuclear PTEN in HCV-replicating cells resulting

from the depletion of transportin-2, which is mediated by a novel viral non-coding RNA, vmr11. In addition, PTEN silencing in Jc1 infected HuH7 cells stimulated HCV particle secretion, while PTEN overexpression decreased virus egress.⁽³⁶⁸⁾ A reduced PTEN function also inhibits cell apoptosis in HCV-infected hepatocytes through activation of the PI3K/Akt survival signaling pathways favoring virus persistence.⁽²⁹³⁾ A recent study showed that the HCV core protein inhibited PTEN at mRNA and protein level in HCV expressing HepG2 cells by activating NF- κ B and the suppression of the NF- κ B activation resumed PTEN activity.⁽³⁶⁵⁾ Also, Cheng et al (2014)⁽³⁶⁶⁾ found that the HCV NS5A protein inhibits PTEN expression at the transcriptional level by decreasing its promoter activity, mRNA transcription, and protein levels in hepatocytes through a cooperation of ROS-dependent NF- κ B and ROS-independent PI3K pathways. However, HCV-mediated PTEN inhibition was not due to a direct effect of the HCV proteins on PTEN since no binding sites of HCV have been found in PTEN promoter.⁽³²⁴⁾ Instead, HCV appears to repress PTEN mRNA translation at post-transcriptional level via PTEN 3'-UTR-dependent mechanisms.⁽³⁶⁸⁾ Non-coding miRs play important roles in protein expression by hybridizing to complementary sites on the 3'-UTR sequences of target mRNAs and thereby inhibiting their translation or triggering their degradation⁽¹⁵³⁾ Thus, it is likely that HCV induces PTEN repression possibly through miR-dependent mechanism.⁽³²⁴⁾ Since PTEN is a direct target of the miR-17~92 cluster, post-transcriptional PTEN alterations during HCV infection could be mediated through activation of the miR-17~92 cluster by the virus.⁽³⁷⁰⁾

Plasma MIR17HG protein and PTEN in HCV-related hepatic inflammation:

In addition to its role during viral infections, the miR-17~92 cluster has a crucial regulatory effect on chronic inflammation and thus, could play a role in the pathogenesis of HCV-related liver injury. In the present study, the plasma levels of *MIR17HG* protein were positively correlated with serum levels of aminotransferases and the METAVIR histological activity grade in patients with chronic HCV infection suggesting that activation of the miR-17~92 cluster was associated with an inflammatory environment in HCV-infected livers. Evidences for an important role of miR-17~92 cluster in the regulation of the inflammatory response have been reported and it was also shown that the expression of miR-17~92 cluster could be triggered by pro-inflammatory cytokines.^(216,237) Trenkmann et al (2013)⁽²¹⁶⁾ showed that TNF- α induces up-regulation of the miR-17~92 primary transcript and its mature miRs (i.e., miR-18a, 19a, 20a, and 92a) in rheumatoid arthritis synovial fibroblasts (RASFs) and they also found that transfection of RASFs with precursor miR-18a revealed significantly increased expression levels of proinflammatory cytokines and chemokines including IL-6, IL-8, macrophage chemoattractant protein-1, and regulated on activation normal T cell expressed and secreted (RANTES). Similarly, transfection of synthetic miR-19b in RASFs resulted in the significant exacerbation of inflammation, as measured through IL-6 and IL-8 spontaneous production. Moreover, another study demonstrated that IL-6 induced miR-17~92 cluster, both in pulmonary arterial endothelial cells⁽²⁰²⁾ and in human hepatocytes and the miR-17/92-derived miR-18a enhanced the acute-phase response in the latter cells through repression of the protein inhibitor of activated STAT-3.⁽²³⁸⁾ Also, Xie et al (2011)⁽³⁷¹⁾ found that the miR-17, miR-20a, miR-19a, were among the inflammation-related miRs that showed increased expression in inflamed periodontal tissues relative to healthy tissues. Also, both in vitro and in vivo assays demonstrated that up-regulation of the miR-17 and miR-20a in macrophages by LPS enhanced macrophage activation, phagocytosis and proinflammatory cytokine secretion through down-regulating SIRP α , an essential signaling molecule that

inhibits leukocyte inflammatory responses.⁽²⁴¹⁾ On the other hand, it has been recently proposed that miR-19a and b, belonging to the miR-17~92 cluster, had a repressive effect on inflammation through targeting TLR2.⁽²⁴⁴⁾ Also, miR-20a, was found to be a negative regulator of inflammation by modulating expression of ASK1, a key component of the TLR-4 pathway, upstream of p38 MAPK.⁽²⁴⁵⁾

The proinflammatory effect of the miR-17~92 cluster could be mediated through targeting of PTEN, which has direct and indirect effects during immune and inflammatory responses. The present study showed a positive correlation between intrahepatic PTEN expression and the METAVIR histological activity grade suggesting a role of PTEN inhibition in hepatic inflammation. It has been documented that PTEN has the ability to suppress T-cell proliferation and survival through its antagonizing effect on the PI3K/Akt pathway.⁽²⁹⁸⁾ A recent study demonstrated that the miR-19b was responsible for promoting Th17 inflammatory responses by repressing the expression of PTEN, thereby augmenting the PI3K-AKT-mTOR axis essential for proper Th17 differentiation.⁽²⁰⁷⁾ Moreover, the miR-19a promoted Th2 cytokine production and amplified inflammatory signaling in both human and mouse T cells by direct targeting of PTEN and Th2 cell responses were markedly impaired in cells lacking the entire miR-17~92 cluster.⁽²³⁴⁾ Furthermore, the induction of the miR-17~92 cluster, specifically miR-18a, targeted PTEN in C3H/HeJ mice, thereby suppressing the induction of the anti-inflammatory CD4⁺Foxp3⁺ Treg cells.⁽²⁴²⁾ Also, PTEN is an inhibitor of the PI3K/Akt/mTOR signaling pathway, which is crucial for site-directed migration and diapedesis of immune effector cells, such as neutrophils and monocytes, to the site of inflammation and infection.⁽³⁰²⁾

Another important mechanism that links the miR-17~92 cluster to inflammation, is the activation of NF- κ B transcription factor, the master regulator of the inflammatory response by inducing the expression of other proinflammatory genes including cytokines, chemokines, adhesion molecules and ROS.^(372,373) The present study demonstrated a positive correlation between the plasma levels of *MIR17HG* protein and the intrahepatic NF- κ B expression which was detected in 90% of patients with chronic HCV infection and was positively correlated with markers of hepatic inflammation. Previous studies demonstrated that NF- κ B expression was up-regulated in HCV-infected livers^(374,375) and HCV core-transfected cells.^(374,376) Also, hepatocellular NF- κ B expression was correlated with necroinflammatory activity (grade) and individual features of portal inflammation, periportal inflammation/piecemeal necrosis and lobular inflammation in allograft liver biopsy specimens in patients with recurrent HCV.⁽³⁷⁷⁾ It has been found that TLR3 senses HCV infection in cultured hepatoma cells, leading to NF- κ B activation and the production of numerous chemokines and inflammatory cytokines, such as regulated RANTES, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , IP-10, and IL-6.⁽³⁷⁸⁾ The HCV core protein activates NF- κ B pathway by increasing the phosphorylation of I κ B α .⁽³⁷⁶⁾ Moreover, it has been found that the HCV NS5A contained one or more NF- κ B binding sites within their promoter region and activation of NF- κ B by NS5A was subsequently demonstrated.⁽³⁷⁹⁾ Also, luciferase assay showed that HCV NS4B activates the multifunctional transcription factor, NF- κ B, in a dose-dependent manner through calcium signaling and ROS and promotes NF- κ B translocation into the nucleus via protein-tyrosine.⁽³⁸⁰⁾ Nevertheless, NF- κ B activation during HCV infection could be mediated through up-regulation of the miR-17~92 cluster by the virus.⁽³⁷⁰⁾

Because miRs have been associated mainly with the repression of gene expression,⁽¹⁵²⁾ the up-regulation of intrahepatic NF- κ B upon activation of the miR-17~92 cluster is most likely due to an indirect effect (*i.e.* the repression of an NF- κ B inhibitor). The miR-17~92 cluster may induce NF- κ B activation via PTEN repression. PTEN inhibits NF- κ B-dependent transcription by blocking the ability of TNF to stimulate the transactivation domain of the NF- κ B p65 subunit.⁽³⁸¹⁾ The present study showed an inverse correlation between intrahepatic PTEN and NF- κ B expression. Moreover, a previous study identified the NF- κ B pathway inhibitor, TNFAIP-3, as a new target of miR-18a using reporter gene assays and thus by repressing TNFAIP3 expression, miR-18a enhances NF- κ B pathway.⁽²¹⁶⁾ Also, Gantier et al (2012)⁽²¹⁷⁾ showed that positive regulation of NF- κ B signaling by miR-19b involves the coordinated suppression of a regulon of negative regulators of NF- κ B signaling including members of the A20/ TNFAIP3-ubiquitin editing complex (A20/ TNFAIP3, Rnf11, Fbxl11/Kdm2a and Zbtb16). In addition, miR-19 inhibits the expression of CYLD, which plays a predominant role in the negative regulation of NF- κ B, by binding to the 3' UTR conservative sites of its mRNA.⁽²⁴³⁾ Furthermore, activation of the downstream Akt by PTEN inhibition facilitates NF- κ B activation resulting in a positive feedback loop. Akt can activate I κ k (I κ B kinase) by phosphorylation, which promotes dissociation of I κ B from NF- κ B, and then induces NF- κ B activity and its translocation into the nucleus.⁽³⁸²⁾ In the meantime, it has been shown that the NF- κ B pathway has a role in the transcriptional regulation of the miR-17~92 cluster. The cluster gene promoter contains 3 NF- κ B binding sites.⁽²³⁹⁾ A recent study showed that the miR-17~92 cluster gene was transactivated through direct binding of NF- κ B p65 subunit to the promoter elements of the cluster in cultured human biliary epithelial cells following LPS stimulation while inhibition of NF- κ B activation by an I κ k inhibitor, blocked LPS-induced up-regulation of pri-miR-17~92 and its corresponding mature miRs.⁽²⁴⁰⁾

Plasma MIR17HG levels and PTEN in HCV-related hepatic fibrosis:

Members of the miR-17~92 cluster have been involved in the development of fibrosis.^(216,247,337,383) In the present study, the plasma levels of *MIR17HG* protein were positively correlated with the METAVIR fibrosis stage in patients with chronic HCV infection and were significantly higher in patients with cirrhosis than in patients with CHC suggesting that activation of the miR-17~92 cluster plays a role in the progression of hepatic fibrosis. In support of these results, Shrivastava et al (2013)⁽³³⁷⁾ observed that the expression levels miR-20a and miR-92a expression in serum were significantly increased in HCV-infected patients with fibrosis as compared to healthy volunteers and non-HCV-related liver disease patients with fibrosis and that the miR-20a expression was positively correlated with the progression of liver fibrosis in HCV-infected patients. Also, Gui J, et al (2011)⁽³⁸³⁾ found that miR 19b was up-regulated 4.3-fold in serum of individuals with cirrhotic livers compared with normal controls suggesting a potentially non-invasive route for diagnosing hepatic fibrosis. Moreover, transfection of RASFs with precursor miR-18a revealed significantly increased expression levels of matrix-degrading enzymes.⁽²¹⁶⁾ In addition, overexpression of miR-17 and miR-20a inhibited senescence in primary human fibroblasts by blunting the activation of p21^{WAF1}.⁽²⁴⁷⁾ On the other hand, it has been shown that effects of the members of the miR-17~92 cluster on fibrosis sometimes have opposite actions. The miR-17~92 cluster could target critical profibrotic genes including CTGF, TSP-1 and TGF- β pathway reducing type-1 collagen production and α 1(I) and α 2(I) procollagen mRNAs.^(249,250,252) Previous studies showed that members of this cluster were

found to be down-regulated in liver,⁽²⁵¹⁾ cardiac⁽²⁴⁹⁾ and idiopathic pulmonary fibrosis.⁽²⁴⁸⁾ The miR-19b had been shown to play an inhibitory role in hepatic stellate cell (HSC)-mediated fibrogenesis through inhibition of the expression of TGF- β RII.⁽²⁵¹⁾ Interestingly, the present study showed that the sensitivity and specificity of the plasma *MIR17HG* protein levels in discriminating patients with cirrhosis from patients with CHC were 100% and 88.9% and in discriminating early fibrosis from advanced fibrosis were 93.8% and 92.9% respectively. These findings may suggest that the plasma *MIR17HG* protein could serve as a potential simple biomarker reflecting the progression of hepatic fibrosis.

The profibrotic effect of the miR-17~92 cluster is mediated, at least in part, via targeting PTEN,⁽²⁰⁷⁾ a negative regulator of tissue fibrosis.⁽³⁰³⁾ The present study showed that the intrahepatic PTEN expression was inversely correlated with METAVIR fibrosis stage and showed a significant decrease in patients with cirrhosis compared with patients with CHC suggesting that PTEN has antifibrotic effects and its loss may play a role in the progression of hepatic fibrosis. Previous studies found that PTEN was down-regulated in the livers of experimental models of hepatic fibrosis.^(305,384-386) Moreover, PTEN-deficient mice exhibited hepatomegaly, fibrosis and eventually developed HCC.⁽³⁸⁷⁾ In addition, Zheng et al (2012)⁽³⁸⁵⁾ showed that the expression of PTEN was reduced with fibrosis in both rat liver tissues and activated HSCs while the reversal of liver fibrosis was associated with increased PTEN expression and the numbers of apoptotic activated HSCs. Also, PTEN expression was negatively correlated with the percentage of α -SMA-expressing HSCs and the severity of hepatic fibrosis in bile duct-ligated mice.⁽³⁰⁵⁾ PTEN has been reported to play a role in the suppression of HSC transactivation.^(382,386) while overexpression of PTEN inhibits HSC activation and proliferation.^(305,386,388) Takashima et al (2009)⁽³⁸⁶⁾ demonstrated that overexpression of PTEN inhibited phenotypic changes associated with HSC activation including morphological changes, expression of α -SMA and α 1(I) collagen, and cyclin D1 expression and also induced apoptosis in HSCs with increased caspase 3/7 activity in isolated rat HSCs. It has been demonstrated that overexpression of PTEN prevented the activation of HSCs into fibrogenic cells through inhibition of the PI3K/Akt pathway.^(386,388,389) It has been reported that PTEN suppression with activation of its downstream Akt pathway provoked liver fibrosis by enhancing α -SMA levels in liver samples from patients with biliary atresia.⁽³⁹⁰⁾ Persistent activation of the PI3K/Akt by down-regulation of PTEN in HSCs enhances cell proliferation, a reduction of cellular apoptosis and ECM production promoting the development of hepatic fibrosis.^(390,391)

Meanwhile, decreased PTEN expression has also been shown to play a role in the fibrosis process in other diseases. In fact, lowered mRNA expression and phosphatase activity of PTEN were found in lung myofibroblasts of patients with idiopathic pulmonary fibrosis.^(304,392) Moreover, PTEN inhibited lung fibroblast proliferation, α -SMA expression and collagen production through inactivation of PI3K/Akt signaling pathway, which was abrogated by a selective PTEN inhibitor.⁽³⁰⁹⁾ A study, also, demonstrated that knocking out PTEN in the cardiac muscle of mice increased heart to body weight ratio, decreased cardiac contractility and resulted in interstitial cardiac fibrosis.⁽³⁰⁶⁾ In addition, loss of PTEN resulted in a 3-fold increase in dermal thickness due to excess deposition of collagen with elevated Akt phosphorylation and increased expression of CTGF, a pro-adhesive matricellular protein, in skin fibroblasts from patients with the fibrotic autoimmune disease; diffuse systemic sclerosis.⁽³⁰⁷⁾ Also, PTEN regulates cell interactions with the

ECM by inhibiting cell migration, spreading, and focal adhesions.⁽³¹¹⁾ It becomes activated during collagen matrix contraction and is responsible for antagonizing PI3K activity and promoting a decline in phosphorylated Akt and fibroblast apoptosis in response to collagen contraction.⁽³¹²⁾ Moreover, PTEN expression decreases MMP-2 expression and activity by a NF- κ B-dependent pathway.^(313,314) Also, PTEN regulates cell interactions with the ECM by inhibiting cell migration, spreading, and focal adhesions.⁽³¹¹⁾ It becomes activated during collagen matrix contraction and is responsible for antagonizing PI3K activity and promoting a decline in phosphorylated Akt and fibroblast apoptosis in response to collagen contraction.⁽³¹²⁾

Plasma MIR17HG levels and PTEN in HCV-related hepatic steatosis:

Hepatic steatosis is strongly associated with HCV infection⁽³⁹³⁾ and is considered an important predisposing factor for liver inflammation, fibrosis, and HCC.⁽³⁹⁴⁾ It has been suggested that HCV alters the expression of genes associated with cholesterol/lipid metabolism.⁽³¹⁵⁻³¹⁸⁾ Meanwhile, HCV-related steatogenesis is required for stable viral replication.⁽³⁹⁵⁾ In infected cells, cytosolic lipid droplets serve as intracellular storage organelles for HCV structural and NS proteins and are essential for virus assembly.^(396,397) However, the molecular mechanism(s) underlying HCV-induced steatosis is not well characterized. In the present study, the grade of hepatic steatosis showed a positive correlation with the plasma levels of *MIR17HG* protein and an inverse correlation with intrahepatic PTEN expression in patients with chronic HCV infection suggesting that the miR-17~92 cluster plays a role in fat accumulation in HCV-infected livers, possibly through down-regulation of PTEN. Accumulating evidence suggested that PTEN is important for the prevention of intrahepatic lipid accumulation and PTEN deficiency in hepatocytes is a critical mechanism leading to hepatic steatosis.⁽³⁹⁸⁻⁴⁰²⁾ Liver-specific PTEN knockout mice sequentially developed steatosis, steatohepatitis, liver fibrosis and HCC.⁽³⁹⁹⁾ Also, the liver of PTEN-deficient mice revealed macrovesicular steatosis, ballooning hepatocytes, lobular inflammatory cell infiltration, and perisinusoidal fibrosis that are characteristic of human non-alcoholic steatohepatitis.⁽⁴⁰⁰⁾ Moreover, PTEN was down-regulated in the liver of rats having steatosis and high plasma levels of fatty acids, as well as in the steatotic livers of patients with NAFLD.⁽³²¹⁾ In addition, acute depletion of hepatic PTEN caused steatosis with increased secretion of hepatic triglyceride and apolipoprotein B.⁽⁴⁰¹⁾

Based on these observations, it is likely that a decreased PTEN expression represents one of the signaling defects promoting steatosis in HCV infection. Previous studies suggested that HCV induces steatosis via PTEN repression. Clément et al (2011)⁽³²⁴⁾ demonstrated that down-regulation of PTEN expression in the livers of HCV genotype 3a–infected patients was associated with the presence of steatosis and caused the accumulation of large lipid droplets in hepatoma cells transduced with the HCV core protein of genotype 3a. Moreover, inhibition of PTEN protein phosphatase activity by HCV stimulated cholesterol metabolism and storage of cholesterol esters in cytoplasmic lipid droplets conferring a replicative advantage to the virus by promoting viral egress from infected hepatocytes.⁽³⁶⁷⁾

PTEN has been shown to alter host cell lipid/cholesterol metabolism and thus induce hepatic steatosis. PTEN deletion stimulates the expression of genes related to lipogenesis in HCV infection.^(315,316) Inactivation (phosphorylation) of PTEN in HCV-infected cell line

with subsequent activation of the PI3K/Akt pathway, mediated the transactivation of SREBPs, major regulators of lipid metabolism that activate genes encoding the synthesis of cholesterol and fatty acids and cellular uptake of lipoproteins.⁽³¹⁵⁾ Also, PTEN inactivation has been reported to impair microsomal MTP activity and contribute to HCV-associated steatosis.⁽³¹⁷⁾ In addition, PTEN directly dephosphorylates HMGCoA reductase, the rate-limiting enzyme in cholesterol synthesis and was increased in HCV-infected livers.⁽³¹⁸⁾ Moreover, PTEN deficiency induced PPAR γ , a key transcriptional activator for adipogenesis and lipogenesis, and its downstream target genes, such as the adipogenic genes (*adiponectin*, *adipsin*, and *aP2*) and β -oxidation-related genes (*acyl-CoA oxidase*, *peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase b*, *functional protein*, and *peroxisomal 3- ketoacyl-CoA thiolase*) and increased the expression of fatty acid-modifying enzymes (*fatty acid synthetase*, *acetyl-coenzyme A carboxylase*, and *steroyl-CoA desaturase 1*).^(319,320) Down-regulation of PTEN expression also affected import, esterification, and extracellular release of fatty acids.⁽³²¹⁾ In addition, PTEN down-regulation leads to constitutive activation of forkhead transcription factors, which mediate abnormal lipid accumulation in hepatocytes.⁽³²²⁾ Furthermore, PTEN is a negative regulator of insulin signaling and peripheral insulin sensitivity⁽³²³⁾ and may contribute to insulin resistance in chronic HCV infection.⁽⁴⁰²⁾ Liver-specific PTEN deletion in mice induced insulin hypersensitivity and enhanced peripheral glucose metabolism in addition to hepatic steatosis.^(321,323,399) PTEN inactivation leads to impaired insulin sensitivity by reducing IRS-1 and inhibition of the insulin signaling pathway through negative regulation of PI3K/Akt signaling pathway.^(321,324)

SUMMARY

Hepatitis C virus infection is one of the main causes of chronic liver disease worldwide. Persistent infection with HCV is associated with the development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Chronic HCV infection is associated with liver cell necrosis, variable degrees of hepatic inflammation and steatosis and is characterized by progressive accumulation of extracellular matrices that contribute to liver fibrosis. The inflammatory response to HCV infection involves the induction of several hundreds of genes, a process that must be finely regulated to achieve virus clearance and to prevent the pathological consequences of a deregulated gene expression. MicroRNAs (miRs) are a family of small non-coding RNAs, approximately 21-24 nt in length, that play a major role in the regulation of gene expression at the post-transcriptional level resulting in gene silencing. Recently, it has been shown that miRs are present not only in tissues but also can be secreted from the cells and can gain access to body fluids (e.g. plasma, serum, urine, saliva and sputum) and can be used as non-invasive, sensitive biomarkers for detecting disease. Most miRs are encoded individually as a single mature miR. However, many miRs are situated in "polycistronic" miR clusters, wherein multiple miRs are generated from the same primary transcript. The miR-17~92 cluster is a prototypical example of a polycistronic miR cluster. This cluster encodes for six mature individual miRs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a). Interestingly, the host gene of the human miR-17~92 cluster, known as the *miR-17 host gene* (*MIR17HG*), encodes a 70 amino acid polypeptide produced by alternative splicing described as the *MIR17HG* protein. Like miRs' components of the miR-17~92 cluster, the *MIR17HG* protein can gain access to body fluids (e.g. blood) and can be used as a simple, non-invasive biomarker for detecting disease. The miR-17~92 cluster acts as a key regulator in a wide variety of biological processes including cell proliferation, apoptosis, metabolism, immune functions, inflammatory responses, angiogenesis as well as organ development. The cluster also activates indirectly nuclear factor-kappa B (NF- κ B) signaling, a potent stimulator of inflammation. The miR-17~92 cluster induces the down-regulation of several targets including phosphatase and tensin homolog (PTEN), a major regulator of the phosphoinositide 3-kinase (PI3K) signaling. It has been found that PTEN deficiency in hepatocytes can induce steatosis and hepatic fibrosis. Also, PTEN loss promotes marked NF- κ B activation and its downstream cytokine pathway.

To achieve this goal, the present work was designed to evaluate the plasma levels of *MIR17HG* protein, encoded by the *miR-17~92 cluster host gene*, and the hepatic expression of its target PTEN in patients with chronic HCV infection in relation to hepatic steatosis, inflammation and fibrosis.

Thirty treatment-naïve patients with chronic HCV infection and 15 age- and sex-matched healthy subjects were included in the study. The diagnosis of chronic HCV infection was based on the following criteria: (1) positive test for HCV antibody; (2) detectable serum HCV RNA and (3) histopathological findings in liver biopsy consistent with the diagnosis of HCV etiology. The diagnosis of cirrhosis was determined by clinical, biochemical and ultrasonographic evidences and histopathological examination when indicated. According to the METAVIR stage, 18 patients were F1-F3 [chronic hepatitis C (CHC)] and 12 patients were F4 [cirrhosis]. Patients with chronic HCV infection were excluded from the study if they had seropositivity for HBV infection; history of alcohol

Summary

consumption; other known causes of chronic liver disease; concomitant schistosomiasis; bleeding diathesis; chronic diseases such as diabetes mellitus, connective tissue diseases or other autoimmune diseases; other infections; any kind of malignancy; cardiac, respiratory or renal disease and previous antiviral therapy.

All patients with chronic HCV infection were evaluated clinically as regards the apparent duration and possible risk factors of HCV infection, symptoms and signs of chronic liver disease. Laboratory investigations were done in all patients and healthy subjects including; complete blood picture and liver test profile [Serum aspartate and alanine aminotransferases (AST and ALT respectively), serum albumin, serum bilirubin, serum gamma glutamyltranspeptidase (GGT) and prothrombin time]. HCV RNA levels in serum were estimated using real time polymerase chain reaction assay. Abdominal ultrasonography was also performed focusing on liver size and echopattern (normal, bright or coarse) and the presence of cirrhosis, ascites and splenomegaly. Quantitative determination of plasma levels of *MIR17HG* protein was performed using enzyme linked immunosorbant assay kit. Core liver biopsies obtained from patients with chronic HCV infection were evaluated according to METAVIR scoring system as regards the histological activity grade (A0-A3) and fibrosis stage (F0-F4). The grade of steatosis was determined by estimating the approximate amount of parenchyma involved as follows: 0 = absent; 1 = mild, less than one third; 2 = moderate, one third to two thirds and 3 = marked, more than two thirds. Immunohistochemical staining of liver specimens was done using antibodies against PTEN and NF- κ B. Positive PTEN immunostaining was scored as follows: score 0: negative staining; score 1: weakly positive staining; score 2: moderately positive staining; and score 3: strongly positive staining. Positive NF- κ B expression was defined as nuclear staining, which could be easily identified at low-power magnification ($\leq 100\times$). Based on intensity of intranuclear staining, the level of NF- κ B intranuclear expression was categorized into four grades: score 0: negative staining; score 1: weakly positive staining; score 2: moderately positive staining; and score 3: strongly positive staining.

Statistical analysis of data obtained from the present study showed the following results:

- The apparent duration of disease ranged between 1-36 months (mean \pm SD = 11.30 ± 10.34 months). The possible risk factors for HCV infection were previous surgery in 6 (20.0%) patients, dental procedure in 5 (16.7%) patients, familial contact in 3 (10.0%) patients and undefined in 16 (53.3%) patients. The presenting symptoms were right hypochondrial pain in 16 (53.3%) patients while none of the patients presented with jaundice, ascites, or previous GI bleeding.
- Ultrasonographic examination in patients with chronic HCV infection showed that the liver was enlarged in 6 (20.0%) patients, normal in 18 (60.0%) patients and shrunken in 6 (20.0%) patients. The liver echopattern was bright in 6 (20.0%) patients, normal in 14 (46.7%) patients and coarse in 10 (33.3%) patients. Splenomegaly was found in the 12 (40.0%) patients with cirrhosis.
- The serum HCV-RNA level in patients with chronic HCV infection ranged between 5.02-7400.00 $\times 10^3$ IU/ml (mean \pm SD = $1333.52 \pm 1752.40 \times 10^3$ IU/ml).

Summary

- The plasma *MIR17HG* protein levels ranged between 19.0-588.0 pg/ml in patients with chronic HCV infection, while the normal values ranged between 17.2- 26.2 pg/ml. Patients with chronic HCV infection showed a significant increase in the plasma *MIR17HG* protein levels compared with healthy subjects (139.69 ± 172.92 pg/ml vs 21.36 ± 2.89 pg/ml; $P = 0.012$). The mean plasma *MIR17HG* protein levels were significantly higher in patients with CHC and patients with cirrhosis than in healthy subjects (34.48 ± 14.95 pg/ml and 297.50 ± 182.21 pg/ml vs 21.36 ± 2.89 pg/ml; $P < 0.001$) and in patients with cirrhosis than in patients with CHC ($P = 0.001$).
- By plotting ROC curve, the sensitivity and specificity of the plasma *MIR17HG* protein levels in discriminating patients with chronic HCV infection from healthy subjects were 83.3% and 100% respectively [Area under the curve (AUC) = 0.957] at a cut-off level of 26.6 pg/ml and in discriminating patients with cirrhosis from patients with CHC were 100% and 88.9% respectively at a cut-off level of 45.5 pg/ml (AUC = 0.995).
- According to METAVIR scoring system, 4 (13.3%) patients showed mild activity (A1), 15 (50.0%) patients showed moderate activity (A2) and 11 (36.7%) patients showed severe activity (A3). As regards fibrosis stage, 3 (10.0%) patients were F1, 11 (36.7%) patients were F2 and 4 (13.3%) patients were F3 stage while 12 (40.0%) patients showed cirrhosis (F4). Steatosis was absent in 12 (40.0%) patients, mild in 8 (26.7%) patients, moderate in 7 (23.3%) patients and marked in 3 (10.0%) patients.
- The sensitivity and specificity of the plasma *MIR17HG* protein levels in discriminating patients with early fibrosis (METAVIR F1 or F2) from patients with advanced fibrosis (METAVIR F3 or F4) were 93.8% and 92.9% respectively at a cut-off level of 40.3 pg/ml (AUC = 0.978).
- Positive immunostaining for PTEN was detectable in all patients with chronic HCV infection mainly as cytoplasmic staining and also as nuclear staining in some patients. The staining intensity was weakly positive in 7 (23.3%) patients, moderately positive in 11 (36.7%) patients and strongly positive in 12 (40.0%) patients. Patients with cirrhosis showed a significant decrease in intrahepatic PTEN expression compared with patients with CHC ($P = 0.012$).
- Positive nuclear immunostaining for NF- κ B was detectable in 27 (90%) patients with chronic HCV infection; of them, 5 (16.7%) patients showed weakly positive staining, 16 (53.3%) patients showed moderately positive staining and 6 (20.0%) patients showed strongly positive staining. There was no statistical difference between patients with CHC and patients with cirrhosis as regards intrahepatic NF- κ B expression ($P = 0.154$).
- Statistical correlations between plasma *MIR17HG* protein levels, intrahepatic expression of PTEN and NF- κ B and other parameters in patients with chronic HCV infection show the following results:

Summary

- No statistically significant correlations were found between plasma *MIR17HG* protein levels, intrahepatic expression of PTEN and NF- κ B on one hand and age, apparent duration of HCV infection and serum HCV RNA levels on the other hand ($P > 0.05$).
- The plasma *MIR17HG* protein levels showed positive correlations with serum levels of AST ($P = 0.014$) and ALT ($P = 0.036$), METAVIR histological activity grade ($P = 0.002$) and fibrosis stage ($P < 0.001$) and steatosis grade ($P = 0.001$).
- There was no significant correlation between plasma *MIR17HG* protein levels and serum GGT levels ($P = 0.850$).
- The plasma *MIR17HG* protein levels were inversely correlated with intrahepatic PTEN expression ($P = 0.002$) and positively correlated with intrahepatic NF- κ B expression ($P = 0.013$).
- The intrahepatic PTEN expression showed significant inverse correlations with serum GGT levels ($P = 0.012$), METAVIR histological activity grade ($P = 0.011$) and fibrosis stage ($P = 0.003$) and steatosis grade ($P = 0.001$).
- There were no significant correlations between intrahepatic PTEN expression and the serum levels of AST ($P = 0.150$) and ALT ($P = 0.216$).
- The intrahepatic NF- κ B expression showed positive correlations with serum levels of AST ($P = 0.009$) and ALT ($P = 0.006$), METAVIR histological activity grade ($P = 0.025$) and fibrosis stage ($P = 0.004$) and steatosis grade ($P = 0.008$).
- There was no significant correlation between intrahepatic NF- κ B expression and serum GGT levels ($P = 0.913$).
- The intrahepatic PTEN expression showed an inverse correlation with intrahepatic NF- κ B expression ($P = 0.006$).

CONCLUSIONS

Based on the results of the present study, it can be concluded that:

- The significant increase in plasma levels of *MIR17HG* protein, encoded by the *miR-17~92 cluster host gene*, in HCV-infected patients indicates that the miR-17~92 cluster is activated in chronic HCV infection.
- The detection of the *MIR17HG* protein using ELISA in blood together with its high sensitivity and specificity in detecting HCV-related liver disease, suggests that the protein could be released from infected hepatocytes into the circulation and thus, could serve as a potential simple blood biomarker for the progression of chronic HCV infection.
- The observation that the plasma levels of *MIR17HG* protein were positively correlated with the enhanced inflammatory response, progression of fibrosis and development of steatosis in HCV-infected livers may suggest that the miR-17~92 cluster is implicated in the pathogenesis of HCV-induced liver injury and fat accumulation in the liver.
- The fact that PTEN is a direct target of the miR-17~92 cluster was supported by the inverse correlation between plasma levels of *MIR17HG* protein and intrahepatic PTEN expression in patients with chronic HCV infection.
- The inverse correlations between PTEN expression and hepatic inflammation, fibrosis and steatosis may suggest that PTEN has anti-inflammatory and anti-fibrotic effects and prevents intrahepatic lipid accumulation and thus, its loss by the miR-17~92 cluster may play a role in HCV-related liver pathology.
- The positive correlation between plasma levels of *MIR17HG* protein and intrahepatic NF- κ B expression indicates that the NF- κ B up-regulation by the miR-17~92 cluster is most likely due to an indirect effect through repression of a NF- κ B inhibitor.
- The activation of NF- κ B by the miR-17~92 cluster and its positive correlation with inflammatory markers, may suggest that NF- κ B plays a role in the inflammatory response mediated by the miR-17~92 cluster in HCV-infected livers.
- Taken together, chronic HCV infection is associated with activation of the miR-17~92 cluster, which plays a role in the pathogenesis of HCV-related liver disease, possibly, through PTEN inhibition and NF- κ B activation.