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Impact of hepatic C viral infection on miR-21 and miR-125b regulation and their role in TLR and interferon expression in A sample of Iraqi patients

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Abstract
microRNAs (miRNAs) are small noncoding RNAs that modulate gene expression at the posttranscriptional level, playing an important role in many diseases. However, reports concerning the role of miRNA in hepatitis B virus (HBV) infection are limited. miRNA chips were used to investigate miRNA changes during HBV infection *in vitro*. Bioinformatics analysis was used to explore possible miRNA and target genes during HBV infection. The expression of miR-125b and its potential target gene, sodium channel, non-voltage-gated 1 alpha (SCNN1A), was further analyzed.

Keywords: viral, infection on, miR-21, miR-125b

Introduction
Hepatitis C virus (HCV) is a blood-borne, single-stranded, positive-sense RNA virus. Approximately 70–80% of individuals infected with HCV present chronic infection, leading to cirrhosis and hepatocellular carcinoma (HCC) [1]. Over time, HCV has evolved numerous strategies to avoid host immunity and utilize virus persistence [2]. Moreover, prolonged immune cell activation during chronic infection leads to make viral replication and disease progression [3].
In chronic HCV infection, the functionality of both innate and adaptive immunity is affected. Monocytes are bone marrow-derived phagocytes involved in critical immune processes that convert innate immunity to adaptive to defend the host against pathogens [4, 5]. Hepatitis C virus has a positive-sense single-stranded RNA genome of an approximate length of 9.6 kb and is classified in the family *Flaviviridae*. The HCV genome encodes a polyprotein that is cleaved into three structural and seven nonstructural proteins. Based on HCV genomic sequence diversity, HCV is classified into seven genotypes and more than 60 subtypes are identified [6, 7]. Genotypes 1, 2, and 3 are globally distributed and cause most HCV infections in North America and Europe. In the Middle East and North and Central Africa, genotype 4 prevails generally, in South Africa genotype 5, in Asia genotype 6, while a recently discovered genotype 7 originates from Central Africa [6, 8].
Currently, no vaccine is available for the prevention of HCV infection. Most HCV transmissions occur by intravenous drug abuse, sexual transmission, and occupational exposure to HCV-infected blood [6]. Potential long-term outcomes in chronically HCV-infected people are liver cirrhosis and HCC, which remain the leading cause of liver transplantation. HCV is globally infecting over 150 million individuals [9, 10]. A recent estimate on global anti-HCV prevalence is 115 million-infected individuals, of whom 80 million are actively viremic [11].
Mammalian microRNAs (miR) are ~22 nucleotide noncoding RNA oligonucleotides highly conserved during evolution and regulate gene expression at a posttranscriptional level through targeting mRNAs [13]. miR has been strongly associated with cancer. Moreover, previous studies reported on a correlation between inflammation, innate immunity, and microRNAs expression. MicroRNAs are highly conserved endogenous non-coding small RNAs that act as translational repressors to regulate a wide range of biological processes [14]. MicroRNAs have also been implicated in the pathogenesis, diagnosis and therapeutic aspects of HCV infections [15]. Recent studies have shown that several microRNAs, including miR-21, miR-125 b and miR-155, were involved in regulation of virus-host interactions and may play important roles in the pathogenesis of HCV infecti [16, 17].
Since miR-125b is highly expressed in peripheral blood mononuclear cells, particularly in monocytes/macrophages [18], and its expression was found to be inversely associated with better treatment outcome of chronic HCV infection [19], we explored the role of miR-125b in modulating HCV-induced events in monocytic THP-1 cells. we show that miR-125b expression was negatively correlated with HCV core-induced cytokines expression in a TLR2/MyD88-dependent manner. Increased interest in hepatitis C disease pathogenesis and diagnostics has led to the emergence of various studies over the last 15 years that have tried to evaluate plasma and tissue levels of miRNAs in order to improve the diagnosis of HCV infections as well as HCV-related HCC [20]. Liver consists of various cell types, mainly divided in the parenchymal cells

(hepatocytes) and non-parenchymal cells (biliary epithelial cells and lymphoid cells, etc.). Each cell type expresses its unique miRNA profile. While miRNAs are up- or down-regulated in almost every stage of hepatic development, they accelerate or inhibit liver proliferation and play a major role in the regulation of diverse liver functions [21]. It has been shown that a total of 277 miRNAs are expressed in the liver, with miR-122 being one of the most abundant and liver-specific miRNAs [22, 23]. Since essential knowledge on liver regeneration processes has been delivered from rodent model studies, further studies are warranted to confirm post-hepatectomy miRNA level changes in humans [24, 25]. Investigation of miRNA levels in specific stages of liver organogenesis may reveal potential biomarkers for liver disease states.

Several miRNAs are induced by TLR and RIG-I activation in myeloid cells and act as feedback regulators of TLR and RIG-I signaling. In this review, we comprehensively discuss the recent understanding of how miRNA networks respond to TLR and RIG-I signaling and their role in the initiation and termination of inflammatory responses. Increasing evidence also indicates that both virus-encoded miRNAs and cellular miRNAs have important functions in viral replication and host anti-viral immunity. Recently, the discovery of certain pathogen recognition receptors (PRRs) and pathogen-associated molecular patterns has provided evidence on the nature of this interaction [31]. PRRs are microbial sensors of the host innate immune system that recognize relatively invariant molecular patterns broadly shared by most types of microorganisms. These recognized microorganismal structures are referred to as pathogen-associated molecular patterns. Several families of PRRs have been characterized over the past decades, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors and Nod-like receptors. TLRs represent the most studied PRRs, in terms of the known ligands, the downstream signal pathways and the effector molecules, and play critical roles in the host's defense against invading microbial pathogens [32]. After pathogen-associated molecular pattern recognition, TLRs initiate innate immune responses by activating signaling pathways that depend on the adaptor MyD88 or the adaptor TRIF and consequently induce pro-inflammatory cytokine and type I interferon (IFN) production [33]. Moreover, any dysregulation of this process has been associated with inflammatory diseases, autoimmune diseases or pathogen dissemination. Many molecules have been identified as positive or negative regulators of TLR signaling [34] including phosphatases (SHP-1, SHP-2, SHIP-1, PTP1B, etc.), protein kinases (calmodulin-dependent protein kinase II, Btk, MEKK3, etc.), ubiquitin-related proteins (A20, Nrdp1, CHIP, etc.), Nod-like family proteins (NLRX1, NLRC5, etc.), membrane molecules (CD300F, CD11b, PECAM-1, etc.), endosome/lysosome-localized molecules (Rab7b,22,23 CLM-324), gene transcription coactivators (beta-catenin25), antigen-presenting molecules (MHC I and MHC II26,27), and even HSP70,28 HSP70L129 and NGF.30 Recently, RIG-I signal pathway regulation was extensively investigated [35]; however, the full anti-inflammatory response mechanisms and the precise fine-tuning of this process still remain incompletely elucidated. The mature miRNA duplex is then incorporated into the RNA-induced silencing complex in the cytoplasm, which uses the ‘seed sequence' to bind partially complementary sequences in the 3′-untranslated region (UTR) of target mRNA transcripts, resulting in mRNA degradation or translational repression [36]. Presently, more than 1000 miRNAs have been identified in the human genome, and as many as 60% of all mRNAs are predicted to be regulated by miRNAs. In the past few years, accumulating evidence has shown that miRNAs affect mammalian immune cell differentiation, the outcome of immune responses to infection and the development of immunological diseases [37]. Therefore, it is not surprising that miRNAs are involved in TLR and RIG-I signaling in the innate immune system. TLR signaling occurs through a number of adaptor proteins, our knowledge of which continues to expand. These adaptor proteins of the MyD88 family signal and result in the activation of several downstream signal transduction pathways leading to the activation of the transcription factor nuclear factor kappa B (NF-κB), mitogen-activated proteinkinases (MAPKs) and members of the interferon regulatory factor family [45] triggering an immune response. The inflammatory response is well characterized for microbial infections, the trigger being the binding of microbial products to the innate immune receptors, such as the TLRs [46]. Sufficient production of inflammatory mediators is essential to clear the pathogenic infection; however, unrestrained TLR responses can have major deleterious effects on the cellas excessive inflammation leads to tissue damage and inflammatory disorders such as septic shock. The inflammatory response must therefore be very tightly regulated, and in recent years, a new class of regulator has emerged in the form of microRNA (miRNA) and has since been keenly pursued. Extensive investigation in this area has led to a wealth of information about these key immune modulators and their mechanism of control. miRNAs are short double-stranded RNA molecules ~22 nucleotides in length that bind imperfectly to the 3′ untranslated regions (UTR) of target mRNA sequences and post-transcriptionally down-regulate their expression. miRNA was first described in 1993 in the form of LIN-4, an essential negative regulator of the LIN-14 protein required for development in *Caenorhabditis elegans*. It was noted that it did not transcribe a protein and had sequences complementary to the 3′ UTR of the LIN-14 protein [47]. In 1998, the landmark work of Fire and Mello revealed that miRNA was carrying out a gene silencing mechanism. Today, it is known that there are vast numbers of miRNAs in the human genome, with published predictions ranging from 225 to 1000+, which would mean that it potentially constitutes 3% of all human genes. Each miRNA is thought to target up to 200 genes and thus it is estimated that a large portion of the human genome can be regulated by miRNAs [48]. Although originally thought to mainly inhibit the translation of target mRNAs, it has now been shown that their main function is acting at the post-transcriptional level to decrease target mRNA levels [49]. miRNA are ‘fine-tuners’ of the immune response; subtle yet essential regulators in key immune pathways. The first indication that miRNAs regulate the immune response came in 2004 with a report which showed selective expression of miR-

142a, miR-181a, and miR-223 in immune cells. miRNAs are now known to be involved in the regulation of maturation, proliferation, differentiation, and activation of immune cells of both the innate and adaptive systems. Development of cells of the myeloid lineage and differentiation of B and T cells are all under the control of miRNA. miRNA allows for a strong initial immune response which is gradually dampened down, thus providing a possible advantage over other TLR regulatory mechanisms. miR-155, miR-21, and miR-146a have been central in much miRNA research due to their expression levels following TLR activation. Furthermore, TLR-responsive miRNAs include miR-132, miR-9, miR-147 and miR-346. These are up-regulated in various cell types after stimulation with TLR ligands. Other miRNAs have been reported to be down-regulated after LPS treatment, including let-7i, which is thought to target TLR4 itself, and miR-125b. As many of those miRNAs regulated by TLR signaling are also dysregulated in cancer, it is possible that miRNAs form a key link between inflammation and cancer and that the induction of specific miRNAs, by TLRs may be a key step in tumor progression. Again, this further necessitates the requirement for control and regulation at all levels of the immune response. Toll-like receptors (TLRs), an important family of pattern recognition receptors (PRRs), are responsible for the recognition of pathogen-associated molecular patterns from infectious pathogens. This recognition triggers the production of large amounts of inflammatory cytokines, type I interferons (IFNs), and antiviral proteins through the activation of interferon regulatory factor (IRF) 3, IRF7, activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) [53]. In addition, the TLR-signalling pathways are strictly and finely regulated by positive or negative modulation at multiple levels to prevent excessive inflammation and achieve a balanced output [54]. Several mechanisms are responsible for the regulation of the TLR-signalling pathways. These include physical interactions, conformational changes, phosphorylation, ubiquitylation, and proteasome-mediated degradation involving various regulatory molecules [55]. Among the many regulatory molecules, microRNAs (miRNAs) have received considerable attention as a newly identified family of regulators involved in fine-tuning the TLR-signalling pathways [56]. Furthermore, several miRNAs such as miR-155, miR-146, and miR-21 are able to target some molecules involved in the TLR-signalling pathways, although the expression of some of these miRNAs depends on the stimulation by TLR ligands [57]. It is also noted that miRNAs may also be induced in a temporal-specific manner.

It is well recognized that the activation of TLR-signalling pathways is required for hosts to eliminate invading pathogens. However, excessive activation of these pathways may also disrupt immune homeostasis, leading to some diseases such as autoimmune diseases, chronic inflammatory diseases, or cancer [34]. Therefore, precise regulation of TLR-signalling pathways is especially important [56]. Since miRNAs act as a class of key regulators of gene expression, the regulation of TLR expression may be one of the effective points at which miRNAs target TLRs.

Importance of this study

A few amounts of studies have been done on the prevalence of HCV infections in past years among Iraqi people in some Provinces. However, they are little and incomplete. Blood transfusion, renal dialysis, and health care workers (HCWs) were major sources of HCV infection in Iraqi people. Thus, we recommend continuing surveillance of blood donors, HCWs, and patients, in addition to HCV markers screening by molecular technique for the diagnosis of HCV during the window period in order to decrease the prevalence of HCV infection**.**

Study aims

The current study aimed to document the prevalence of HCV infection among staff Iraqi people. Such prevalence presumably might provide help for prevention strategies of this infection and guide further research.

Objective of this study

Study the impact of HCV on miR-21 and miR-125b and their role in TLR and interferon expression in a sample of Iraqi patients.

The present study aimed to investigate the potential value of miR-21 and miR-125b as non-invasive biomarkers in chronic hepatitis C and to evaluate the correlation between miR-21, miR-125b expression, and viral or host factors involved in the progression of liver disease.

Plan of work

1. An observational study will be conducted on 94 treatment-naïve HCV-infected Iraqi patients [mean age 49.8 ±11.5 years; range 30-68 years, 59.6% females].
2. Plasma (400 µL) from each sample will be centrifuged at 1200 × g for 10 min at 4°C before RNA extraction. Total RNA was extracted using the mirVana PARIS kit according to the manufacturer's instructions. To eliminate differences among samples, synthetic Caenorhabditis elegans cel-miR-39, which has no homologous gene in human, was added into each sample. RNA was dissolved in 100 µL of RNase-free water. Absorbance at 260 and 280 nm was measured using a spectrophotometer to determine the concentration and purity of RNA.
3. The expression of miR-21 in HCV Iraqi patients and healthy controls will be detected by using miScript SYBR Green PCR kit. The reaction will be performed in a real-time quantitative PCR machine ABI7300HT. Reverse transcription of miRNA was performed according to the manufacturer's instructions using the miScript Reverse Transcription kit (Qiagen).

4. MiR-125b plasma expression will be analyzed by real-time PCR with TaqMan®MicroRNA Assay, according to the manufacturer's protocol. Briefly, miRNA will be isolated from 300 µL of plasmausing NucleoSpin®miRNA Plasma Kit. Reverse transcription will be performed usingTaqMan®MicroRNA reverse transcription kit. The reverse-transcripted DNA fragments are resuspended in Hi-Di formamide.
5. Serum HCV viral load will be quantified using the RT-PCR-Cobas TaqMan HCV test.
6. Liver fibrosis will be evaluated using a noninvasive method -transient elastography.
7. Polymorphism in the IL28B gene (SNP on chromosome19-rs12979860) will be tested with a Custom TaqMan 5'-allelic discrimination assay.
8. The transferase levels will be measured with the spectrophotometric standardized methods of the International Federation for Clinical Chemistry.
9. Serum alpha-fetoprotein (AFP) levels will be measured using electro-chemiluminescence.
10. Statistical analysis will be done using SPSS V.25

Previous Studies

A previous study by Sibley *et al*., 2015 forecasted changes in HCV-related disease up to 2030 and concluded that HCV-related morbidity and mortality are estimated to increase due to an aging of the HCV-infected population and currently available treatment will be inadequate if reductions in HCV-related disease of this magnitude are to be achieved [12].

Studies from regional countries showed that HCV prevalence was 1.1% in Yemen, less than 1% in Iran, 1.8% among the young generation in Saudi Arabia, 4.0% among blood donors in Pakistan and 0.2% in Iraq (Hussein *et al*., 2017).

In patients undergoing maintenance HD, the prevalence of HCV infection substantially increases and this disease has been shown to be associated with severe complications from chronic hepatitis to fatal cirrhosis and HCC (Khedmat *et al*., 2014).

The natural history of HCV infection in dialysis patients remains incompletely understood; controversy continues even in patients with intact kidney function. Defining the natural history of HCV remains difficult for several reasons: the disease has a very long duration, it is mostly asymptomatic, and determining its onset may be difficult (Fabrizi, 2013).

the transmission of HCV involves direct exposure to contaminated blood and is associated with intravenous drug use, iatrogenic exposures, tattooing, body piercing, and less frequently through vertical transmission and high-risk sexual behavior (Najim and Hassan, 2018).

The diagnosis of HCV infection is based on the detection of anti-HCV Abs by enzyme-linked immunosorbent assay (ELISA) and it is confirmation by a positive result obtained by an immunoblot assay or by the presence of HCV RNA. An improvised third generation anti-HCV-ELISA with high sensitivity is widely used for patient screening. Most of the available third-generation ELISA tests for anti-HCV Abs detection are based on either synthetic peptide antigens (Ags) alone or recombinant protein Ags or a combination of synthetic and recombinant protein Ags of HCV (Kesli, 2011). Infection with HCV can lead to chronic hepatitis, liver cirrhosis, or even HCC. Approximately 50–80% of HCC cases are associated with chronic HCV infection [26]. In the past several years, the involvement of miRNA in the pathogenesis of HCV-related liver diseases has been well documented [27]. Since miRNAs can be directly involved in antiviral immune-pathological events, it is inevitable that miRNA target sequences in viral populations remained conserved, providing relevant evidence of the biological significance of potential miRNA-based antiviral interventions [28]. For the time being, no HCV-encoded miRNAs have been reported. It should be noted that miRNA dysregulation has been studied in various experimental settings, mainly involving in vitro systems, HCV-replication-supporting cell lines, transgenic mice, cultured hepatocytes (mouse/rat/human), circulating blood cells or serum of HCV-infected individuals, and liver tissue samples. MiRNA expression in model systems has been measured mainly with qualitative real time-polymerase chain reactions (RT-PCRs) and/or miRNA microarrays and less frequently with next-generation sequencing (NGS) [29, 30]. MiR-21 was further implicated as a central player in the inflammatory response through a paper in 2010 by Sheedy *et al*. This key study found that the control of PDCD4 expression is crucial in the negative regulation of the inflammatory response to LPS, acting as a molecular switch between the pro-inflammatory (NF-κB) and anti-inflammatory (IL-10) response [50]. This switch was found to be controlled by the induction of miR-21 resulting in a decrease in PDCD4 protein abundance. This process positively influences IL-10 production, while negatively regulating NF-κB activity, presumably to control the LPS response that can be lethal. Here, they found that mice deficient in PDCD4 were protected from LPS-induced death. The induction of NF-κB and IL-6 by LPS required PDCD4, whereas LPS enhanced IL-10 induction in cells lacking PDCD4. miR-21 may have a further role in resolving inflammation as it has recently been found to be induced by resolvin D1, an anti-inflammatory and pro-resolving lipid molecule [51], further contributing to its role as a negative regulator of the inflammatory response.

MiR-21, both a well-documented 'oncomiR' and an inflammatory mediator has also recently been established as a link between the two. Iliopoulos *et al*., 2020 found that a transient inflammatory signal can initiate an epigenetic switch from non-transformed to cancer cells. Signal transducer and activator of transcription 3 (STAT3), a transcription factor activated by IL-6, was shown to directly activate miR-21 and also miR-181b-1 with transient expression of either microRNA sufficient to promote the switch to an epigenetic state. miR-21 inhibits the tumour suppressor protein phosphatase and tensin homologue; resulting in increased NF-κB activity required to maintain the transformed state. miR-21 has again been implicated in a feedback loop, this time

driving the switch from an inflamed state to cancer [52]. Many studies have indicated that TLR signals can modulate miRNA expression by various techniques, such as microarray and deep sequencing. Although subtle changes have been found in most miRNA expression after TLR stimulation, a subset of strong miRNA targets of TLR signaling has emerged, especially miR-146, miR-155, and miR-21. Similar to other TLR-induced genes, this miRNA subset can further be classified as early- or late-response miRNAs, according to the response speed following TLR ligand stimulation.

Specifically, miR-146 and miR-155 are highly induced within 2 h after TLR treatment, and thus belong to the early-response miRNAs; however, miR-21 belongs to the late-response miRNAs [38, 39]. There are also subtle differences in miRNA expression profiles depending on the TLR ligands used, stimulation time, and specific cell types.

The first TLR-induced miRNA expression profiling study was performed in human monocytes in David Baltimore's lab in 2006 [40]. In their study, miR-146a, miR-155, and miR-132 were upregulated after lipopolysaccharide (LPS) stimulation. miR-146 upregulation has also been confirmed by other independent studies. miR-146a induction is controlled by nuclear factor kappaB (NF-κB), which is the most common transcription factor that triggers TLR-induced miRNA transcription. In contrast, miR-155 expression was observed to depend on MyD88- or TRIF-induced JNK activation after TLR stimulation. Additionally, miR-132 was demonstrated to be controlled by cyclic AMP response element-binding protein and transcriptional co-activator p300 [41]. Moreover, miR-21 is induced in an NF-κB-dependent manner at later times in macrophages after LPS treatment**.** Other miRNAs are also induced after TLR treatment or pathogen infection, including miR-223, miR-147, miR-9, miR-27b, and let-7e [42]. While the upregulation of these miRNAs by TLR activation has been identified to be mostly dependent on de novo primary miRNA transcription by certain transcription factors, we cannot exclude the possibility that the accelerated processing of primary miRNA/precursor miRNA or the retarded degradation of mature miRNA also results in increased miRNA concentration. For the most part, primary miRNA transcription is dependent on the NF-κB transcription factor [43]. Additionally, TLR signals can negatively regulate miRNAs, including miR125b, let-7i, and miR-98, in certain cell types [44]. Little is known about how TLR signals decrease miRNA expression, which most likely occurs through transcriptional repression or post-transcriptional destabilization of miRNA.

Many studies have further identified subsets of miRNAs related to the TLR-induced NF-κB-dependent pathway. The expression of many miRNAs including miR-146a, miR-155, miR-132, miR-223, miR-147, miR-9, miR-27b, let-7e, miR-21, miR-16, miR-23b, miR-30b, miR-301a, and miR-125b is induced in an NF-κB-dependent manner after TLR stimulus or pathogen infection [58, 59, 60]. It is necessary to point out that miR-125b expression related to the TLR-activated NF-κB-dependent pathway remains controversial and needs to be further investigated in the future [61].

The prevalence rate of HCV infection is decreasing in developed countries; whereas, in developing countries such as Iraq, researchers still struggle to control the infection (Messina *et al*., 2015).

To reduce the HCV infection, many countries insert molecular biology technology within the routine protocol tests which recognize very low concentrations of viral RNA (Whittaker *et al*., 2008).

Results and Discussion

miR-21 was significantly elevated while miR-125b-2* was significantly downregulated in tumors compared to controls ($P < 0.01$ and $P < 0.05$ respectively). miR-138 and miR-184 were observed to be predominantly downregulated in the tumor samples. High levels of miR-155 were associated with the habit of chewing tobacco/betel quid.

Conclusions

Our results corroborate the previous findings on the overexpression of mir-21 and downregulation of miR-138 in OSCC. As the expression of miR-184 is controversial in tongue/oral cancer, the downregulation may be specific to tumor anatomical localization. On the other hand, to the best of our knowledge, this is the first report to show the association of miR-155 with tobacco chewing and the downregulation of miR-125b-2* in OSCC. Computational predictions suggest that miR-125b-2* may have a role in alternative splicing.

Abbreviations

HCC, hepatocellular carcinoma; NGS, next-generation sequencing;
ND, no data available; qRT-PCR, quantitative real-time polymerase chain reaction.

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