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Hepatitis C: Host and Viral Factors Associated with Response to Therapy and Progression of Liver Fibrosis

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Abstract

The goal of this study was to identify the baseline host and viral factors of response to antiviral therapy in patients with chronic hepatitis C. Compared with interferon/ribavirin therapy, new current direct-acting antiviral (DAA) combination regimens significantly increased rate of sustained virologic response (SVR) and shorter treatment durations, but is still limited by viral resistance, adverse effects, and high cost especially in developing countries. Human genetic factors and heterogeneity within the HCV genome may be associated with virologic treatment failure before and after antiviral therapy. Further, HCV infection may contribute to the development of HCV-related liver disease and hepatocarcinogenesis, through modulating genetic and epigenetic state of certain genes implicated in control of critical cellular pathways. Previous results confirm the importance of host and viral factors and virus-induced genetic and epigenetic changes in predicting outcome and treatment response.

Keywords: hepatitis C virus, response to therapy, IFN-based antiviral therapies, direct-acting antiviral (DAA) agents, genetic variability, liver fibrosis, hepatocarcinogenesis, epigenetic changes

1. Introduction

Chronic hepatitis C virus (HCV) infection with the estimated worldwide prevalence of 1.1% is a global health problem, affecting 170 million people worldwide [1, 2]. About 15–45% of infected persons spontaneously clear the virus without any treatment, but remaining 60–80% will develop chronic HCV infection leading to fibrosis, cirrhosis and/or hepatocellular

carcinoma (HCC) [1, 3]. Previous results suggest that, worldwide, there were 1.75 million new HCV infections and annually an estimated 700,000 persons with chronic HCV die untreated [1]. Until 2014, the standard of care to treat HCV infection was pegylated interferon and ribavirin (PEG-IFN and RBV). However, first generation of (DAAs) NS3/4A protease inhibitors (PI) were boceprevir and telaprevir, administered with PEG-IFN and RBV [1]. These protease inhibitors were limited by viral resistance, adverse effect and long treatment duration and high cost especially in development countries [4]. The standard care of therapy is changing rapidly and new (DAAs) therapy such as sofosbuvir, daclatasvir, and the sofosbuvir/ledipasvir combination are part of the preferred regimens in the WHO guidelines with achieve cure rates above 95%. In comparison with older therapies, new therapy is more effective, safer, and better-tolerated with shorter treatment (usually 12 weeks), but high prices of the medicines limit the expansion of HCV in many countries [1]. However, challenges remain in optimizing current drug regimens, limiting the problem of resistance mutations and promote individual therapy [5–7]. Treatment predictors are important factors for management of therapy in patients with chronic hepatitis C infection.

1.1. Host and virus-related factors associated with response to therapy

Previous studies indicated that baseline host and virus related factors such genotype, viral load, age, gender, stage of liver fibrosis, and *IL28B* (interleukin-28B) polymorphisms were associated with therapy outcome [8–16]. Among viral factors, HCV genotype and baseline level of HCV-RNA are significant determinants of treatment outcome. Sustained virologic response (SVR) was defined as undetectable levels of HCV RNA, 24 weeks after cessation of treatment. Rapid virologic response (RVR) was defined as undetectable levels of HCV RNA at 4 weeks. Early virologic response (EVR) was defined as undetectable levels of HCV-RNA at week 12 (complete EVR) or ≥ 2 log reduction in HCV viral load from baseline (partial EVR), while non-response NR was defined as detection of serum HCV RNA 6 months after cessation of treatment. Thus, RVR is a strong predictor of SVR, but absence of an EVR is significant predictor for non-response (NR) to antiviral treatment. Moreover, viral kinetics during therapy provides information on how to individualize treatment [16, 17]. It is known that stages of liver fibrosis may be associated with response rates to PEG-IFN/RBV therapy. Further, patients with advanced liver fibrosis (METAVIR score F3-F4) were more frequently in non-responders (NR) than in patients with minimal or mild fibrosis (F0-F2), especially in patients with genotype 1 [8, 12, 18]. According to the previous results, infection with genotypes 2 or 3, younger ages, lower baseline viral load, and absence of advanced fibrosis were all strong predictors of SVR. Since 2011, therapeutic regimens for HCV genotype 1 patients were modified. Combination of NS3/4a protease inhibitors and pegylated interferon and ribavirin improved the SVR rates [19]. However, boceprevir- and telaprevir-based regimens are associated with side effect and lower efficacy than the newer DAA therapies. Also, this therapy is effective only in patients with genotype 1 [1, 20]. With respect to host and viral factors and first generation DAAs, viral kinetics is the most important predictive factor of SVR. The *IL28B* was associated with greater chances to shorten therapy but there is no correlation with SVR [8]. Second-generation DAAs have higher rates of SVR, are safer and can be used in combinations that obviate the need for interferon and ribavirin [1]. However, failure to new DAAs combinations is in association with patients with

poor response, genotypes 1a or 3, advanced liver cirrhosis, elevated level of viral load and the presence of human immunodeficiency virus (HIV) coinfection [21]. In contrast, some authors suggest that therapy outcomes are not significantly influenced by *IL28B* polymorphisms, HCV genotype, high baseline viral load, or prior interferon failure [22]. In the era of DAAs, surveillance of HCC after eradication of HCV by antiviral therapy is particularly important. Current new therapies with DAAs are associated with high rates of SVR, generally exceeding 90% even among patients with cirrhosis or prior treatment failure. Therefore, understanding of various host and viral factors associated with disease progression and development of HCC in chronic hepatitis C infection is important for implementing personalized treatment. One of the most interesting current questions concerns the impact of DAAs on HCC incidence [23].

1.2. HCV-host interactions and host genetic alterations

In the specific environment of every host, the outcomes of the HCV infection will be different. There are many factors which influence the therapy outcome and progression of liver disease. These factors include baseline clinical and pathohistological parameters. Also, host genetic landscape has effects on therapy outcomes and development of liver disease phenotypes. Genome-wide association studies (GWAS) have been created to track genetic polymorphisms in the human genome which associate with, virus clearance, therapy outcomes, and different stages of liver disease. Thus, this kind of the genome screening could be a useful tool in the diagnostics and the therapy of hepatitis C, and could lead to a personalized therapy.

1.2.1. Role of interferons (IFN) in HCV infection

HCV infection induces production of interferons λ (IFN- λ). IFN- λ s bind to IFNL receptors (IFNLR) activating JAK-STAT signaling pathway which induces expression of ISGs (interferon-stimulated genes). The IFNLR is a heterodimer, consisting of two subunits, IL10R2 and IL28RA. There are four IFN- λ s described so far: IFNL 1–4. The gene loci for these proteins are located on the chromosome 19. Study of GWAS has revealed polymorphisms in IFNL gene loci, which are in association with HCV clearance, either spontaneous or therapy-induced. Previously it was shown that single nucleotide polymorphisms (SNPs) near *IL28B* (rs12979869 and rs8099917) were strongly associated with response to PEG-IFN/RBV therapy in patients with genotype 1 and with spontaneous virus clearance [24–27]. In the neighborhood of *ILR3*, a novel dinucleotide polymorphism, ss469415590 (TT/ Δ G), has recently been discovered which is in high linkage disequilibrium with rs12979869 and participate in formation of a novel gene *IFNL4*. The *IFNL4* gene creates IFNL4 protein, but TT variant of ss469415590 does not form the protein [28]. The TT variant has been shown to be beneficial to its carriers because it influences the spontaneous and therapy-induced virus clearance [26]. Among people of African ancestry, the polymorphism ss469415590 is in stronger association with virus clearance than the rs12979869 [28]. There are two variants of IFNL4 protein with impact on antiviral activity, which differ in only one amino acid at the place 70. The carriers of the variant with serine (P70S) have better therapy response and higher spontaneous virus clearance rate compared to carriers of the variant with proline. However, the expression of ISGs in the variant P70S is decreased, which is in discrepancy with its better antiviral activity. The researchers have

speculated this is probably due to decreased adaptive immunity as the consequence of high expression of *ISGs* in carriers of IFNL4-P70 variant [28]. The genes of human leukocyte antigen (HLA) family located on the chromosome 6 are associated with clearance of HCV infection. Some authors found that this connection is inconsistent, because of different systems of HLA typing, clinical phenotypes, and ethnic backgrounds. The only polymorphisms of *HLA* genes which are confirmed so far to have an association with virus clearance are HLA-DQB1*03 and HLA-DRB1*11 [29–32]. The receptors of natural killer (NK) cells Killer-cell immunoglobulin-like receptors (KIR) and their corresponding ligands, HLA class 1 proteins, also have a role in the immune response. Studies of the association of the polymorphisms of KIR and HLA-C and virus clearance have given controversial results. In some studies, the carriers of a KIR2DS3 (killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 3) and homozygosity for *HLA-C1* were associated with spontaneous virus clearance [33, 34]. Polymorphisms of some other genes coding for the proteins which have a role in the immune response have also been shown to correlate with spontaneous/therapy-induced virus clearance. One of these genes is the gene for osteopontin which initiates T helper cells type 1 response, and has been shown to associate with the sustained viral response after the IFN therapy [35, 36]. Today, the role of the host genetic variability has been reduced due to high efficacy of new era interferon-free antiviral therapy. However, the high cost of this therapy limits its availability to the developed parts of the world.

1.2.2. The influence of host genetic alterations on the progression of liver fibrosis in chronic HCV infection

On the other hand, the host genetic variability has a significant role in the formation of different liver phenotypes. The phenotypes, such as fibrosis, steatosis, cirrhosis, or hepatocellular carcinoma (HCC) are the consequence of virus infection and disease progression. The polymorphisms of the immune response genes have effect on disease progression or inhibition. For example, the patients with chronic hepatitis C, which carry rs12979860CC variant, have higher fibrosis progression rate. This effect is even more pronounced in the younger females and in the carriers of the HCV type 3 [37]. The polymorphisms of apoptosis-related genes *MERTK* (MER proto-oncogene, tyrosine kinase), *TULP1* (tubby like protein 1) and *RNF* (ring finger proteins) gene family have association with the progression of the HCV-related fibrosis [38] and the genes of the major histocompatibility complex (MHC) with cirrhosis [39] and HCC [40]. However, these results need confirmation in the further research. Although having some potential, GWAS studies have many flaws. One of them is the impossibility of the screening of more extensive genetic changes, e.g., *copy number variation* (CNV) and epigenetic events, which also take part in the disease progression and the therapy response. Methodological failure is that these studies use conservative significance thresholds to eliminate false positive signals and many significant polymorphisms of low frequency could be unregistered, as reviewed in [41]. All discovered polymorphisms so far are currently not applicable to the clinical classification of the liver disease, because of their low predictive value. One of the possible solutions is the formation of the polygenic scores. For example, there is an earlier study which resulted in the formation of cirrhosis risk scores (CRS) based on the gene signature of seven genes [42]. In another study, a prediction model for liver fibrosis

was based on gene polymorphisms. This model includes polymorphisms of *IFNL* and clinical risk factors, which taken together give a risk for liver fibrosis [43]. Based on the fibrosis/cirrhosis risk score, therapy decisions can be made. Chronic inflammation and cirrhosis of hepatic cells lead to HCC. Hepatocellular carcinoma is the one of the deadliest type of cancer in the world. It represents a heterogeneous disease consisting of many tumor subpopulations with different frequencies of mutated genes. There are several genes which are affected, during the HCC progression, either by genetic or epigenetic alterations. In recent studies, the alterations of the *TERT* (telomerase reverse transcriptase), *CTNNB1* (catenin beta 1), *TP53* (tumor protein p53), and *AXIN1* (axin 1) genes are shown to correlate with tumor progression [44, 45]. *TERT* promoter mutations have been present in 64% of HCV-related HCC [44]. *TERT* is a subunit of the telomerase enzyme, whose mutations lead to telomere shortening and uncapping of chromosomes, which then leads to chromosome fusion and general chromosomal instability. *TERT* mutations and silencing of *CDKN2A* (cyclin dependent kinase inhibitor 2A) gene by promoter hypermethylation are the events that coincided [46]. It seems that *TERT* mutation is an early event in the cancerogenesis [45]. Frequent *CTNNB1* mutations have also been observed in HCV-related HCC [46]. There were attempts to classify HCC based on genetic signatures. The genetic signature represents one gene or a collection of genes, with characteristic expression profile, which is confirmed to be specific for the diagnosis, prognosis, and treatment response prediction [47]. Genetic signatures could be the means of classifying HCC in the groups which would facilitate diagnosis and therapy decisions. In the first attempt to make a molecular model for a diagnosis of an early HCC in the HCV patients, the best accuracy was achieved using the signature of three genes *LYVE1* (lymphatic vessel endothelial hyaluronan receptor 1), *GPC3* (glypican 3), and *BIRC5* (baculoviral IAP repeat containing 5). The *TERT* gene and *E-cadherin* were also shown to be informative in that model [48]. One European study revealed molecular signatures of 243 HCCs, based on fibrosis and cirrhosis score, and various risk factors, among which HCV was present in 26% of the cases. However, this study did not find any associations of these molecular signatures with HCV infection [45]. Cancer Genome Atlas Research found 26 frequently mutated genes in HCC. They identified *ALB* (albumin), *APOB* (apolipoprotein B), *LZTR1* (leucine zipper like transcription regulator 1), *EEF1A1* (eukaryotic translation elongation factor 1 alpha 1), *SMARCA4* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4), *AZIN1* (antizyme inhibitor 1), *RP1L1* (retinitis pigmentosa 1 like 1), *GPATCH4* (G-patch domain containing 4), *CREB3L3* (cAMP responsive element binding protein 3 like 3), *AHCTF1* (AT-hook containing transcription factor 1) and *HIST1H1c* (histone cluster 1 H1 family member c) gene mutations. Additionally, this study used methylation profiles, based on which the HCC were divided into four methylation clusters. The fourth cluster was characterized by high frequency of *CTNNB1* and *TERT* mutations, *CDKN2A* promoter hypermethylation and presence of HCV infection [46]. Besides smaller genetic changes, the changes which encompass bigger regions of DNA such as deletions, insertions, copy number variations, loss of heterozygosity also have a role in the HCC progression. All of these changes alongside hyper and hypomethylation lead to general chromosomal instability, which is prerequisite for tumor progression. There are examples of the direct influence of HCV on host genomic instability. HCV protein NS5A interacts with host gene *ASPM* (abnormal spindle microtubule assembly), which regulates mitotic spindle formation, causing interruption of the cell cycle, eventually

leading to chromosomal instability and HCC [49]. HCV Core protein induces polyploidy by decreasing of retinoblastoma-associated protein expression, thereby influencing mitotic cycle checkpoint, which also leads to chromosomal instability [50]. In addition, interaction of NS3/4A with serine protein kinase, which has a role in the cell cycle, leads to disabling cell repair system [51]. The polymorphisms in the host genome have effect on therapy response, virus clearance, and differentiation of HCV-induced liver phenotypes. The genetic signatures in the host genomes could be the means of therapy decisions making, and a way to personalize therapy. However, some other factors, such as epigenetic events and alterations of larger DNA regions, should also be taken into an account.

1.3. Impact of hepatitis C virus heterogeneity on therapy outcomes

The persistence of hepatitis C virus (HCV) infection and its poor susceptibility to treatment have been attributed, at least in part, to the high rate of genetic variability exhibited by the virus.

The persistence of HCV infection and its poor susceptibility to treatment is the consequence of high rate of virus genetic variability. Variability of virus is due to the low fidelity of viral RNA-dependent RNA-polymerase, which lacks proof-reading capacity, thus allowing the generation of quasispecies. Genetic variability is segregated within particular segments of the HCV genome, resulting in a number of highly variable regions [52]. These quasispecies play an important role in the escape from selective pressures by immune responses and antiviral therapies. A decrease or no change in the number of nucleotides substitutions in the protein kinase (PKR) binding domain (PKRBD) and the interferon-sensitivity-determining region (ISDR) were associated with failed to respond to PEG-IFN/RBV treatment [53]. It was reported that the presence of >4 mutations in the PKRBD region of NS5A protein was correlated with SVR to peg-IFN/RBV therapy [54]. Moreover, PKRBD sequences might be used as a prognostic guide for treating HCV-1-infected patients [54, 55]. Absence of substitutions at positions 70 and 91 in Core protein is a significant predictor for the success of IFN-based therapy [54]. On the other side, amino acid substitutions in the Core protein play an important role in early dynamics of viral replication during IFN-based therapy in chronic HCV infection and more frequent occurrence HCC. With respect to new DAAs therapy, efficacy of NS5A inhibitors can be blocked by presence of NS5A resistance-associated substitutions (RASs), but choice of DAA regimen and duration of therapy depends on multiple viral and host factors.

2. Epigenetic changes in HCV-induced liver diseases

Persistent infection with HCV is associated with the development of chronic liver diseases: fibrosis, cirrhosis and ultimately, HCC [56, 57]. Prevention of chronic hepatitis C and its complications is based on antiviral therapy and early detection of reliable molecular markers in persons under the risk [57, 58]. However, current antiviral therapies are not effective in many patients with chronic hepatitis C, so there is a need for a greater understanding of the factors leading

to progression to HCC in order to design novel approaches to prevention of HCV-associated complications [59]. Here, we discuss the current knowledge about the inter-relationship between HCV and pathophysiology of HCV-associated chronic liver diseases, with particular focus on the virus-induced host epigenetic changes leading to hepatocarcinogenesis.

2.1. Hepatocellular carcinoma

According to epidemiological evaluation, HCC is the fifth most common cancer and the second most common cause for cancer death in the world [60]. Although the prognosis of patients with HCC has marginally improved over the last few decades, the five-year survival rate remains poor as a result of late diagnosis. Consequently, the majority of patients with advanced HCC do not survive for longer than 6 months from the time of diagnosis [61]. It has been shown that in chronically infected patients, the risk of developing HCC is strictly correlated to fibrosis stage, and the incidence of HCC is more frequent in patients with liver cirrhosis than in those with mild fibrosis [62]. SVR to IFN-based therapies decreases HCC incidence in a large number of HCV patients, indicating the importance of eradicating the virus to prevent carcinogenesis [63, 64]. However, despite a successful virus clearance, the risk of HCC still exists in individuals with severe fibrosis and continuous HCC monitoring is recommended [65]. So, it is of great importance to determine molecular markers of progressive fibrosis, that could indicate the chronically HCV infected persons under the risk of developing HCC [66, 67]. HCV-induced hepatocarcinogenesis is a multifactorial process which results from a complex interaction among host, environmental, and viral factors [67]. It is considered that at least three host cellular pathways are affected in this process: cell cycle, proliferation, and apoptosis [68]. As HCV is an RNA virus with limited integration of its genetic material into the host's genome, it was first assumed that its ability to transform hepatocytes is linked to indirect mechanisms. Chronic inflammation induced by viral infection results in a permanent degenerative and regenerative processes and occurrence of progressive fibrosis and cirrhosis [69–71]. In addition, chronic inflammation leads to increased levels of reactive oxygen species (ROS), which damage hepatocytes and can lead to accumulation of genetic and epigenetic alterations in hepatic cells [67, 72]. All these events can promote neoplastic transformation of hepatocytes and the progression of malignant clones [73]. Later studies have shown that HCV is directly involved in hepatocarcinogenesis, through direct action of viral proteins on host tumor suppressors and proto-oncogenes [74, 75]. Several viral proteins have been shown *in vitro* to possess functions that could favor hepatocarcinogenesis, through inducing genetic and epigenetic alterations. In particular, the Core protein, NS3, NS4B and NS5A can transform various cell lines, either alone or in cooperation with oncogenes [76–80]. These proteins interact with a number of host factors and signaling pathways leading to the progression from chronic hepatitis C to liver cirrhosis and HCC [68].

2.1.1. Epigenetic changes in HCV-induced HCC

Many lines of evidence suggest that aberrant epigenetic changes associated with viral infection may trigger events that promote the neoplastic transformation of hepatocytes [59, 73]. Epigenetics is defined as heritable state of gene expression without altering DNA sequences. Epigenetic mechanisms include genomic DNA methylation, chemical modifications

of histone tails, and non-coding miRNA regulation [81]. Epigenetic changes play a critical role in control of cellular processes through switching genes on and off, thus leading to differential expression of proteins [82]. HCV infection has been shown to induce or correlate with some epigenetic changes that may contribute to HCV-related liver diseases, including hepatocarcinogenesis [68]. It has been proven that certain HCV-encoded proteins induce promoter methylation of multiple genes, thereby affecting their expression [83, 84].

2.1.2. Host genes promoter methylation induced by HCV

DNA methylation represents the addition of methyl group (CH₃) to a fifth carbon of cytosine residues within a CG dinucleotide, frequently referred to as cytosine-guanine dinucleotide (CpG). DNA methylation is an essential component of epigenetic machinery that regulates transcriptional state of many genes. Methylated promoters often lack transcriptional activity, which could result in gene inactivation. As a transcriptional regulator, DNA methylation has a considerable impact on the development of many cancers, including HCC [81, 85]. Aberrant promoter hypermethylation of tumor suppressor genes involved in the cell proliferation, apoptosis, cell adhesion, DNA repair, and detoxification is frequently detected in HCC, resulting in loss of the corresponding gene function [86, 87]. It is believed that changes in DNA methylation patterns are early events in hepatocarcinogenesis and they can even occur at the early stages of HCV induced liver fibrosis [88]. This is supported by the results of the study conducted by Zekri et al., in which they demonstrated that methylation of certain host genes increase with liver disease progression, from fibrosis to HCC [89]. Moreover, the same group of authors has been shown that methylation of certain tumor suppressor genes affect the response to the antiviral therapy [90]. Considering this, a better understanding of methylation changes and how they correlate with disease progression will help in finding novel biomarkers for early detection of HCC and its prevention.

2.1.3. HCV-encoded proteins inducing host genes methylation

It has been demonstrated that HCV Core protein up-regulates levels of DNA methyltransferase (DNMT) 1 and 3b and induces promoter hypermethylation of tumor suppressor genes like *p16* (*CDKN2A*) and *E-cadherin* [91, 92]. Consequent inhibition of p16 expression results in inactivation of pRb (retinoblastoma protein) and subsequent activation of E2F transcription factor 1 (E2F1), which lead to growth stimulation of hepatocytes. Inactivation of *p16* tumor suppressor gene, that regulates cell cycle, appears to play an important role in the pathogenesis of HCC. It has been demonstrated that reactivation of p16 by transferring the *p16* gene can inhibit the proliferation and reduce the invasive ability of HCC cells [93]. Down-regulation of *E-cadherin* by Core-induced hypermethylation leads to epithelial-mesenchymal transition, cell detachment from the surrounding matrix, and migration outside of the primary tumor site, which is known to be a critical event during the late stage of carcinogenesis [94]. Besides these, methylation of some other tumor suppressor genes, like suppressor of *SOCS-1* (cytokine signaling 1), *GSTP1* (glutathione S-transferase pi 1), *APC* (adenomatous polyposis coli), and *RASSF1A* (Ras association domain family member 1), has been detected in HCV-associated HCC compared normal liver [95, 96]. Abnormal promoter methylation of most of these genes

was detected in the plasma/serum DNA as well as in the tissue DNA of HCC patients, which gives opportunity for designing noninvasive blood tests for detection of methylation markers and to distinguish HCV patients who will eventually progress to advanced stages of fibrosis [97]. Zhang et al. reported that the analysis of methylation status of *RASSF1A*, *p16*, and *p15* (cyclin-dependent kinase inhibitor 2B, *CDKN2B*) in serum DNA of infected people could be a valuable biomarker for early detection of HCC in the populations at high risk, including chronic HCV infection [98]. Iyer et al. recorded high frequencies of *p15*, *p16*, *APC*, *FHIT* (fragile histidine triad), and *E-cadherin* promoter methylation in the plasma and liver tissue of HCV-associated HCC patients, with high concordance for all examined genes [99]. Zekri et al. have shown that methylation of *MGMT* (O-6-methylguanine-DNA methyltransferase) gene can be used as a predictor of response to the antiviral therapy, while *RASSF1A* methylation status could be a marker of fibrosis severity [90]. As authors reported, promoter methylation of *MGMT* gene appeared at higher frequencies in the NR than in the responders, which was explained by the fact that *MGMT* has an important role in protecting cells against DNA damage, via triggering DNA repair mechanisms [100]. On the other hand, the same group of authors has shown that *RASSF1A* methylation was significantly higher in HCV patients with mild fibrosis, which support the role of an intact *RASSF1A* gene in inducing the fibrogenesis in chronic HCV patients [90]. Another study by Hayashi et al. reported that HCC patients with SVR have different molecular alterations compared to NR with continuous HCV infection [101]. This group of authors observed lower frequencies of *p16*, *RB1* (RB transcriptional corepressor 1) and *PTEN* (phosphatase and tensin homolog) genes promoter hypermethylation in patients with SVR, while methylation of *p15* and *p14* (ARF tumor suppressor) genes was not detected in this group of patients, compared to those with the present HCV infection. Interestingly, *p16* methylation was detected with the highest frequency in the both groups, suggesting important role of *p16* gene in the development of SVR-HCC. Authors speculated that *p16* in hepatic stem cells might be methylated in the continuous presence of HCV. These cells with methylated *p16* gene might survive and grow after eradication of HCV by IFN therapy. In addition, despite an improved understanding of mechanisms leading to HCV-induced HCC and development of highly potent antiviral therapy, HCV-related HCC remains a global health problem. The development of valuable molecular biomarkers will be of a great importance to distinguish a group of HCV infected people with a high risk for hepatocarcinogenesis. Based on the studies conducted so far, genomic DNA methylation could function as a non-invasive, sensitive, and specific biomarker for prediction the response to the antiviral therapy and early detection of HCC.

2.2. MicroRNA biogenesis and function

MicroRNA (miRNA) are non-coding genetic elements participating in the regulation of gene expression by RNA interference in plants and animals, while in human cells, are associated with viral infection, as well. According to The Encyclopedia of DNA Elements (ENCODE), approximately 75% of the human genome transcribes into a various types of RNA molecules, coding and non-coding [102, 103]. Among non-coding RNA (ncRNA), miRNAs emerged as biologically, physiologically, and clinically significant, considering the fact that they silence translation of partially complementary target messenger (mRNA) molecules. MicroRNAs participate in the regulation of gene expression by RNA interference. In the last 15 years,

miRNAs were linked with various types of diseases and disorders, and majority of investigations were based on the changes in their expression levels. MicroRNAs are encoded by a miRNA gene, which transcribes into an immature-primary microRNA (pri-miRNA) molecule, a double stranded, hairpin-like genetic structure. Then, two enzymes-RNase endonuclease III Drosha, and DGCR8 (molecular anchor part of a microprocessor complex), transform pri-miRNA into a 70 nucleotides (nt) long precursor microRNA (pre-miRNA) in the nucleus [104]. The process of miRNA maturation occurs in the cytoplasm, where Dicer or Argonaute (Ago), and other protein-partners, cooperators cleave pri-miRNA into a 22 nt long miRNA, ready to be recognized by RNA-induced silencing complex-RISC [105, 106]. The recognition and binding of miRNA "seed" sequence to 3' untranslated region (3'UTR) of target mRNA incompletely complementary with "seed" region at miRNA molecule results in either translational repression, or mRNA degradation. Translational repression and degradation result in the decrease of protein levels, thus changing genetic, biological, and physiological processes, activity of various signaling pathways. So, main characteristic of miRNAs is to regulate amounts of synthesized proteins [107]. miRNAs are present in every human cell, and in body fluids (as circulating), such as serum, plasma, urine, saliva, and even gingival liquid. MicroRNA circulates through the body via body fluids as free-circulating miRNAs, and packed into the exosomes and vesicles, as exosomal miRNAs. Extracellular, exosomal circulating miRNAs may carry over the information about disease progression, infection status, and other clinic pathological parameters of HCV infection and HCC formation and progression, but it is still not completely clear. Liver-specific miRNAs such as miR-122 and miR-192, may be released from damaged liver cells, providing the information about liver. Exosomal and circulating miRNAs, as well as miRNAs extracted from tissue may be involved in intercellular communication. Besides the fact that nearly 300 miRNAs are expressed in normal, healthy liver, miR-122 represents the major fraction of liver-specific miRNAs, together with miR-192, miR-199a/b-3p, miR-101/99a, and members of oncogenic let-7 family [108].

2.2.1. MicroRNA in HCV infection and HCC

MicroRNAs are described as onco miRNAs, some of them as tumor suppressive, and several of them even have dual role in cancer pathogenesis and presumably in other physiological processes and pathological condition [109]. During the HCV infection, onco miRNAs such as miR-21/155/221 activate and might cause formation and facilitate progression of HCC. On the other hand, the decrease of some tumor suppressive miRNAs during the HCV infection might also cause hepatocarcinogenesis, such as miR-198 [110]. Firstly, some miRNAs directly interact with the genome of HCV. Secondly, several miRNAs are potential biomarkers of the presence or progression of the HCV infection. Thirdly, several miRNAs are indicators of HCC formation and/or progression. Fourthly, there are miRNAs associated with HCC genotype and clinicopathological characteristics of infected patients and histopathological characteristics of tumors, while some miRNAs such as miR-134/320c/483-5p may be used as early biomarkers for HCV infection in the future. Finally, some miRNAs, such as miR-122 represent potentially great targets for future therapeutics in the aspects of treatment of HCV-associated liver diseases and HCC. Large numbers of different studies have recently been focused on miRNAs in a different points related to different points of HCV infection, i.e., replication of the virus, viral

genotype, response to HCV therapy, lipid status, liver function indicators, stage of liver fibrosis, HCC grading, and response to chemotherapy. Changes in the expression levels of miRNA several micro RNAs such as miR-21/122/134/141/155/192/199/221/320c/373/483-5p/491/758, let-7b, etc., are associated with different stages of the viral life cycle and the progression of infection [111]. Circulating miRNAs, miR-122, and miR-222 have been shown to be valuable as potential future diagnostic tool for HCV infection within the Egyptian patients [112]. Upregulated miR-10a/15a/17-5p were associated with HCV-related HCC, miR-122 was characterized as potential diagnostic tool for HCC within HCV-infected individuals, while overexpression of miR-221/222-3p was characteristic exclusively for HCV-related HCC [108]. MicroRNA 122 is a liver-specific miRNA, a regulator of HCV tendency to infect hepatic cells. miR-122 is crucial for efficient HCV infection and viral spread and speed up the replication of the virus in hepatocytes, and facilitates viral protein synthesis [113]. Besides miR-122, as the mostly studied miRNA in HCV-HCC patients that recognizes two different sites at HCV 5'UTR, increased levels of miR-448 and miR-196 attenuate HCV replication by binding to the Core and NS5A sequences of the HCV genome [114]. Secondly, some miRNAs, such as miR-199a have opposing characteristics during the HCV infection. Namely, miR-199 targets sequences of HCV genome and blocks the transcription of HCV RNA [115]. Thirdly, during the HCV infection, miR-155 is usually up-regulated. Higher levels of the well-known onco-miRNA, miR-155 induce proliferation of hepatocytes, increasing the chance of HCC formation [116]. For example, changes in the expression levels of miRNAs such as miR-134/320c/483-5p were shown to be significantly higher within the HCV-infected individuals, compared with healthy controls. In our previous article, we described heterogeneity in behavior of microRNA in cancer studies [109, 117]. Another evidence is supporting our observation on the importance to notice how level changes of the particular miRNA can be important for one event, while having no significance for another similar event, related to the same type of the disease or pathological condition, showing high specificity of some miRNA molecules. Namely, miR-20a/92a levels investigated in the sera of the patients having HCV-related liver disease were associated with the disease severity, and the higher grade of liver fibrosis, while levels of the same miRNAs have not shown any association with grade of liver fibrosis and other pathological characteristics examined within the patients with non-HCV-related liver diseases. Another example of involvement of miRNAs in various segments of HCV-HCC pathology, HCV proteins change levels of miR-193a that results in reduction of sensitivity to chemotherapy of HCC patients [118]. Furthermore, in our article related to the heterogeneity of miRNAs. According to several researches, it has been shown that miR-122 may be anti-tumorigenic properties in mice knockdown studies [119].

2.2.2. MicroRNA as future therapeutics for HCV and HCC

Considering the fact that several miRNAs can modulate expression of up to a dozens or even hundreds of target genes, it is not surprising that miRNA-based therapy is still challenging. Nevertheless, this multi-targeting ability also represents an advantage for future miRNA-based therapeutics. There are two major approaches for future miRNA-related therapeutics miRNA inhibition (Lock nucleic acid (LNA) anti miRNAs, antago miRNAs, miRNA zippers, small molecules inhibitors of miRNAs, and miRNA sponges) and miRNA substitution strategies (miRNA mimics and miRNA vectors), with the purpose to either silence miRNA activity

or to replace absent miRNA molecules [109, 110]. Several studies proposing that miRNA panels could be used in near future as biomarkers for screening of the HCV-related liver diseases. The changes in their expression levels were associated with staging of liver disease progression and anti-HCV therapeutics [108]. Probably, manipulation with several miRNAs at the same time might be crucial in treatment of HCV-HCC. For example, inhibition of upregulated miRNAs involved in viral replication, such as miR-122, simultaneously with miRNAs, in combination with inhibition of miRNA such as miR-155 which promotes cancerogenesis, or mimicking miRNA whose under expression helps HCV replication, and mimicking down-regulated miRNA with tumor suppressive function in HCC.

3. Nomenclature

The Family: Flaviviridae, Genus: Hepacivirus, species: Hepacivirus C.

(Guidelines of the International Committee on Virus Taxonomy (ICTV) <https://talk.ictvonline.org/>)

4. Conclusions

Chronic HCV infection ultimately leading to HCC will remain a global health problem in the coming decades. Despite increasing knowledge regarding mechanisms of HCV-induced HCC, prevention of HCV-induced HCC is not yet fully established. So, it is important to define both viral- and host genetic and epigenetic patterns on the onset of infection and in early and advanced stages of inflammation and fibrosis in order to predict response to the antiviral therapy, and thus avoid potential complications of persistent HCV infection. Further, for screening a population and making a correct diagnosis about the presence of infection, it is necessary to use standardized commercial tests with universal values and analyze homogenous group of patients. Despite new DAAs therapy, this virus remains unbeatable. When the heterogeneity of the HCV virus, the host genetic and epigenetic variability, and the differences in the therapy outcomes are taken into account, the only right way to fight this disease is personalized therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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