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REVIEW

Insights for hepatitis C virus related hepatocellular carcinoma genetic biomarkers: Early diagnosis and therapeutic intervention

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Abstract

The current review explores the role of emerging molecular contributing factors in liver carcinogenesis on top of hepatitis C virus (HCV). Here we will try to discuss the role genetic and epigenetic factors in pathogenesis of hepatocellular carcinoma. Understanding the role of these factors will help in discovering the mystery of liver carcinogenesis on top of chronic HCV infection. Moreover, use of the studied molecular factors will provide the hepatologists with tailored diagnostic promising biomarkers and flatten the way for establishment of emerging molecular treatment based on exploring the molecular subscription of this aggressive liver cancer.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Genetic; Epigenetic; Diagnosis

Core tip: It was evident that pathogenesis of hepatocellular carcinoma (HCC) among cases with hepatitis C virus (HCV) infection results from interaction between viral factors and host factors. The host factors include genetic and immunologic factors. Identifying the emerging genetic factors which are contributing in pathogenesis of liver cancer is considered as revolution in research fields of genetics and oncology. Detection of early promising diagnostic biomarkers and development of specific therapy for HCV related HCC is the hope of most researchers in the related fields.

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INTRODUCTION

Primary liver cancer is an increasing malignant disease,

being one of the most important causes of cancer deaths all over the world[1]. The journey from hepatitis C virus (HCV) infection to hepatocellular carcinoma (HCC) development takes 20-40 years while in some people it may take few years. This variable progression may refer to host factors that interfere; accelerate; delay or even stop HCC development.

Liver fibrosis is the corner stone in the process of hepatic carcinogenesis through course of chronic HCV infection. In cases with liver cirrhosis, the newly discovered cases with HCC are 1%-7% per year, although HCC does not usually develop in livers with early stages of fibrosis[2,3]. Recently, emerging efficient antivirals for chronic HCV infection as sofosbuvir is used to decrease the opportunity of liver carcinogenesis[4]. Surprisingly, completely cured cases could not guarantee the avoidance of liver cancer development, particularly cases with late stages of liver fibrosis[5,6].

Underlying genetic mechanisms of HCC caused by HCV have not been fully understood. Clinical evaluations indicate that the main task of HCV in liver cancer is to make a cirrhotic tissue background for liver carcinogenesis[7].

Hepatitis B virus can integrate into genetic material of hepatocytes leading to mutation and liver carcinogenesis. The situation in cases of chronic HCV infection is different; hepatocarcinogenesis develop due to direct effects of viral particles or through indirect way which is initiation of chronic hepatitis, liver fibrosis and cirrhosis[8].

Scientists usually face a big challenge to explore the exact underlying mechanism for HCV related liver fibrosis and hepatocarcinogenesis due to the shortage of the ideal animal model for chronic HCV infection. HCV infection is restricted to human and chimpanzees. In a trial to do the researches on tissues which closely resemble that of human, some scientists used treated and modified animal models as HCV transgenic mice and immunocompetent humanized mice and they succeeded to detect known sides of chronic HCV infection natural history. We are still in need of an ideal animal model that can illustrate the chronic HCV infection and its complications as liver tumorigenesis[5,8].

In the current review we explore some of the underlying molecular contributors for liver cancer development in cases with chronic HCV infection. These molecular players may act as promising early detectors or even an emerging therapeutic target for HCC tailored therapy.

ONCOGENIC EFFECTS OF HCV PROTEINS

Development of HCC on top of HCV occurs due to contribution of viral and host factors. HCV can induce HCC through direct effects of its protein or through indirect way. The indirect way occurs as inflammation of liver tissue and/or its complication as cirrhosis which form the background for HCC in most of HCV - HCC patients. Hoshida *et al*[9] described HCV as a single-strand RNA virus in the Flaviviridae family that encodes structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The virus is established by a nucleocapsid containing viral genome; core protein and envelope glycoproteins E1 and E2. HCV infection induces the expression of the nucleocapsid core protein by infected cell. This core protein centralizes in the cytoplasm, lipid droplets, endoplasmic reticulum/Golgi apparatus, nuclei and mitochondria this expression is assumed to alter several cellular functions.

ONCOGENIC EFFECTS OF CORE PROTEIN

Previous studies concluded that HCV core protein can cause apoptosis, signal transduction, share in oxidative stress by producing reactive oxygen species (ROS), affect metabolism of lipid, activate transcription, and modulate immunity and transformation[10,11]. Many scientists have reported frequent mutations in the core gene of HCV among subjects with liver cancer[12,13].

Some scientists suggested that p53 and p73 are tumor suppressor proteins can be affected by HCV core protein[14,15]. HCV core protein binds with p73 to inhibit p73 α-dependent cell growth arrest in a p53 - dose dependent manner. This was supported by findings of Alisi *et al*[16]. Furthermore, study of Yamanaka *et al*[17] suggested that core can also modify the major target of p53 which is known as the cyclin dependent inhibitor p21WAF1 and could control functions of cyclin/cyclin-dependent kinase complexes included in cycle of cell and control and carcinogenesis. This was agreed by study of Kwun *et al*[18]. Moreover, other researchers found that signaling pathways such as Raf/MAPK[19], Wnt/β-catenin, 41 and TGF-β can be stimulated by HCV core protein[20,21]. The inclusion of different pathways is known to be activated in HCC and may help in progression of cirrhosis process; induce mutation for one or more oncogenes or tumor suppressor genes[22].

ONCOGENIC EFFECTS OF NS3 PROTEIN

HCV NS3 protein acts as an early oncogenic player on hepatocytes[23,24]. It inhibits the activity of both p53 and p21WAF1 promoter[25,26]. Meanwhile, NS3 protein promotes cell growth, DNA-binding functions of the reproduction agents, AP-1 and ATF-2 and JNK activation[27]. it was found that HCV NS3 can activate AP-1 and NF-kB to increase production of TNF-α which has a role in liver cercinogenesis[28].

ONCOGENIC EFFECTS OF NS5A PROTEIN

Hassan *et al*[27,28] proved that NS5A is necessary for replication of the virus and is present in the cytoplasm of infected hepatocytes in conjunction with endoplasmic reticulum. NS5A shares in many function of the cell as transcription, transformation, signal transduction, ROS production and apoptosis. Interestingly, wild-type *NS5A* gene was up regulated among HCC patients with liver

cirrhosis as background Compared with those who did not develop HCC, taken together, irregular data with regard to the function of core and NS5A proteins on hepatocytes signaling pathways, transcriptional activation, apoptosis and lipid metabolism oxidative stress propose a varied role for HCV proteins in the pathogenesis of chronic hepatitis due to HCV infection, liver fibrosis that results in liver tumorigenesis[28].

INFLAMMATION-RELATED LIVER CARCINOGENESIS

The indirect way for hepatocarcinogenesis during HCV infection is inflammation of hepatocytes, persistence of chronic hepatitis, liver fibrosis and cirrhosis ending to malignancy transformation. In liver cancer, close to 80% of patients develop malignancy on top of chronic hepatitis. However, the underlying genetic changes for HCC development are not yet fully understood. Continuous formation of regenerative nodules in liver cirrhosis shares in malignant transformation. Previous study reported activation of toll like receptor 4 which promotes the effect of translocation of intestinal microbiota to the liver in late stages of liver carcinogenesis[29]. In this study, Dapito *et al*[29] used animal model (*i.e.,* TLR4 genetic inactivation, gut sterilization and long-term treatment with low doses of lipopolysaccharide (LPS), in which chronic liver injury was modeled using diethyl nitrosamine and carbon tetrachloride.

The researchers proved that the NF-kβ pathway is stimulated through identification of TLRs for microbial ligands, like LPS and pathogen-related molecular manner. As a result, the secretion of inflammatory molecules, such as TNF-α and cytokines is stimulated. These molecules regulate the function of liver cells particularly stellate cells which act as the maestro for liver fibrosis process, a step that forego liver cancer growth[30,31]. The findings of this study support that of other studies who concluded that the main predisposing factors for HCC development among cases with chronic HCV infection is late liver fibrosis and cirrhosis[2,3,7].

GENETIC CHANGES DURING HCV RELATED LIVER CARCINOGENESIS

Moeini *et al*[31] suggested that human cancer diseases have been hallmarked by the acquisition of cancer cells to six capabilities: (1) growth signals self-adequacy; (2) loss of sensitivity to anti-growth signals; (3) escaping from apoptosis; (4) unlimited possibility for replication; (5) continuous formation of new blood vessels for the tumor; and (6) metastasis[32]. A growing line of evidence has shown that aberrant expression of miRNAs is included in different cancer diseases through deregulating target genes, collectively leading the cell to acquire the six capabilities. miRNAs can act as oncogenes, tumor suppressors, or both and this depends on the targeted genes[33].

Changes in Genetic and epigenetic represent host factors for HCC pathogenesis in late stages of HCV infection. Several signaling Mediators are contributing in liver carcinogenesis, involving some control cell differentiation (Hedgehog, WNT, and Notch), signaling for growth factor (*e.g.,* HGF, IGF, PDGF, EGF, FGF,) and angiogenesis (VEGF). Intracellular modules as AKT/MTOR and RAS could share in pathogenesis of HCV related HCC. Other genetic causes are contributing to stimulate erratic pathway activation. These include mutations, chromosomal abnormalities, and epigenetic mechanisms[34].

Heterogeneity and complexity of carcinogenesis has altered the way we believe concerned with induction, pathogenesis, diagnosis, progression and management of cancer. Although the great advance in exploring of cancer biology, the most of emerging therapies for malignancy do not achieve efficient success, which points to failure of conventional therapeutic interventions. The corner stone in applying of an emerging effective treatment against malignancy is the establishment of efficient clinical trials. Invasive surgical procedures and liver transplantation, the important procedures for HCC therapy, are considered to be the most curative options for treatment of cases of liver cancer. But the frequent recurrent HCC and metastasis after surgical approach is the main hurdle in HCC treatment. Applying effective curative therapeutic procedures to late stages of HCV-HCC disease usually faces big challenge. So that, detection of HCC as early as possible is the corner stone in raising the survival rate and improving the prognosis for cases with this aggressive disease. A main attempt to promote novel treatments should involve the implementation of genetic identification to describe tumors and supply exact foretelling as possible therapeutic targets during the process of liver carcinogenesis and an overall improvement in targeted therapies.

Insights of genetic profiling implying the development of HCC on top of HCV are obscure. The molecular mechanisms include up regulation of oncogenes, inhibition of malignancy oppressor genes, up regulation of growth agents[35], stimulation of telomerase and DNA mismatch repair error may share in the development of liver carcinogenesis[20,36,37]. In this context, over expression or down expression of the studied genes which are related to cell cycle progression, growth, disease creation, and reaction to surrounding stimulants cooperate leading to this sophisticated process.

The genomic alterations in malignancy performs a constitutional signature which could involve the control through transcriptional pattern which in turn reflect on a quantitatively gene expression levels[38,39].

Moinzadeh *et al*[40] reported that the implementation of high technologies analysis are so paramount important to improve exploring of genomic alterations in the situation of its relation to pathogenesis of HCC; with the preface of copy number variation (CNV) notion in addition to single nucleotide polymorphisms (SNP), and with the amended mapping of such CNVs throughout the

whole genome of cases *vs* healthy subjects. In the same concept, Zhao *et al*[41] proved that CNVs as chromosomal SNPs that are several megabases in size, is ending with the size range of CNVs proportionate with the great progress in bioinformatics. The identification of these polymorphisms, either at small (SNPs or mutations) or large CNVs scale as well as regions contains loss of heterozygosity (LOH) blocks may have a role in cancer formation.

EPIGENETIC ALTERATIONS IN HCV RELATED HCC

Epigenetics refers to all stable alterations in gene expression with no underlying modifications in the genetic sequence itself[42]. Epigenetic and genetic mechanisms have a role in silencing of key cellular genes leading to destabilization of the genome and in turn resulting in carcinogenic transformation in human cancers, including HCC[43]. Contribution of different epigenetic factors, including genomic DNA methylation, histone modifications, and miRNA regulation, contribute to HCC dissemination, invasion, and metastasis. The reversal of deregulated epigenetic changes has emerged as a potential strategy for the treatment of HCC and is of paramount important in preclinical and clinical development[44]. However, obtaining a highly-specific potent epigenetic markers may provide an opportunity for targeting inflammation-epigenome cross-talk in HCC and needs employment of fast screening methods, such as high-throughput screening to navigate efficiently and discovering epigenetic targets[45].

Administration of classical antiviral agents, INF administration with epigenetic drugs (such as DNMT inhibitors or HDAC inhibitors) could confirm an efficient counteracting between cytokines and epigenome changes[46]. It was reported that HCV core protein could increase the expression of mRNA and protein values of DNMT1 and DNMT3b, which in turn leads to epigenetic alteration of liver cells of patients with in HCV cells infection[47].

Furthermore, the induction of HCV proteins or the infection of HCC cells with HCV cell culture (HCVcc) resulted in suppression of histone H4 methylation/acetylation and histone H2AX phosphorylation, with significantly altered expression of genes essential for HCC development, indicating that HCV-induced overexpression of PP2Ac involved in pathogenesis of HCC through deregulation of epigenetic histone modifications[48]. HCV infection may up regulate histone deacetylation activity through affecting hepcidin expression, a key suppressor of iron availability[49]. The induced HCV oxidative stress leads to suppression of hepcidin expression by increased histone deacetylase function.

Other epigenetic changes during HCV induced liver carcinogenesis is deregulation of a class of short, non-coding RNAs [microRNA (miRNA)] that play important roles in gene expression regulation. One miRNA can target several genes through mRNA, this function put miRNAs in the top not only of diagnostic markers but some of them became a target for personalized therapy. They act as genetic signature for many diseases including HCC with different stages, supporting the potential use of miRNAs in HCC patient stratification of diagnosis and prognosis. Several studies suggested that miRNAs play an important role in carcinogenesis, either as oncogenes or tumor suppressors[50].

Interestingly, miRNAs have been found to be differentially expressed in liver cancer, they are activated to share in pathogenesis of HCV related HCC. Moreover, some miRNAs could be related to different stages of liver carcinogenesis, supporting the possible use of miRNAs in HCC patient correspondence to diagnosis and prognosis. Some of these HCC-associated miRNAs have been validated in independent cohort studies. This confirms the ability of paving the way to develop HCC diagnosis, evaluation of risk exposure, and patient danger accordance with the eventual aim of tailored treatment.

Several previous studies have identified miRNAs expression in pathogenesis of liver cancer on top of chronic HCV infection. miR-21, miR-17, miR-222, miR-224 miR-221, are usually increased in liver cancer[51,52] while miR-200, let-7, miR-29, miR-123, miR-122, miR-199a, miR-199b, are decreased[53,54], miR-199 is consistently down-regulated in HCC[55]. SincemiR-199a/b-3p suppresses HCC in part by preventing the p21-stimulated kinase 4/Raf/MEK/ERL pathway, down-regulation of miR-199a/b is related to bad prognosis and low survival rate[56]. On the other hand, miR-224 has been found to be increased in liver cancer[57] and was reported to be related to malignancy aggression, deteriorated liver function, and poor prognosis[58].

BIOMARKERS FOR HCC EARLY DETECTION

Cancer diagnostics based on measuring biomarkers in tissue samples has already in the past decade provided revolutionary advances in diagnosis, prognosis, and therapy selection. A major drawback of the tissue-based approach centers on the need for invasive surgical procedures in sample collection, which in a great many instances preclude following the progression or regression of disease during therapy.

In recent years, an impressive number of cancer biomarker researchers have turned their attention to the demonstration of markers present in biological fluid or blood based biomarkers have also significantly impacted approach of "molecular pharmacogenomics and therapeutics"[59,60]. Deep understanding of pathogenic evolution of cancer has improved considerably through Launching of molecular diagnostics in the marketplace and involves expertise in managing resources and navigating a competitive environment. Rising healthcare costs have led to innovative solutions which include molecular testing matched with targeted therapies, point-of-care testing to provide rapid results for improved

patient outcomes, and non-invasive testing options[60].

Screening for HCC among Patients with chronic HCV infection should be done by conventional abdominal ultrasonography; serum α-fetoprotein (AFP); protein induced vitamin K absence-Ⅱ and abdominal computed tomography scan. Other serum markers could be used as AFP-L3 (a glycosylated form of AFP); Des gamma carboxyprothrombin and Golgi membrane protein 73, Dickkopf-1[61], and squamous cell carcinoma antigen[62] have increased the chance for early HCC detection. We face challenge in diagnosis of small tumors or in well-to-moderately differentiated HCC as serum markers are rarely elevated. Thus, development of sensitive and specific diagnostic biomarkers became an urgent need. It was found that use of autoantibody to tumor-associated antigens (TAA) as a diagnostic biomarker for early detection as indicators of disease prognosis has been explored. Hong *et al*[63] investigated the serum autoantibodies to TAA, and detect that centromere protein F, and hot shock protein were new promising early detectors for HCC. Anti-TAA antibodies might reflect molecular events associated with tumorigenesis.

AFP

The first serologic assay for diagnosis and clinical follow-up of patients with liver cancer was AFP which has been the conventional tumor biomarker for HCC for many years. Serum AFP levels are often increased in HCC, but this is not always the case. AFP levels may be elevated initially in the early stages of HCC and then drop or even normalize before increasing again as disease progression occurs[64]. Total AFP can be divided into three different glycoforms, AFP-L1, AFP-L2, and AFP-L3-based on their binding capability to lectin Lens culinaris agglutinin. High percentage of AFP-L3 has been shown to be associated with poor differentiation and biologically malignant characteristics, worse liver function, and larger tumor mass[65].

mRNAs circulating biomarker

The advantage of circulating nucleic acids in plasma offers another avenue for noninvasive monitoring of a variety of physiological and pathologic conditions[66,67]. Numerous applications based on the detection of circulating cell-free nucleic acids in human plasma have been reported for the management of malignancies. Cell-free plasma RNA detection methods offer an opportunity for the development of pathology-related markers[68,69]. From cell free mRNAs HCC biomarkers are the AFP mRNA, gamma-glutamyl transferase mRNA, insulin-like growth factor Ⅱ, and Albumin mRNA.

Now, accumulating studies have addressed that biomarkers are validated components of tumor pathogenesis. Different biomarkers that better predict patients who are at higher risk of recurrence and shown poorer prognosis would help guide the alternative treatment[70-83]. Despite the investigation of curative or palliative treatments, prognosis is still poor due to underlying liver diseases and the unique biology of HCC.

RNAi biomarker: Derives its attractiveness as a therapeutic tool from several factors. Its principle based on its extreme specificity, the ease of siRNA synthesis, low cost of production and chemical stability makes RNAi an attractive candidate for therapeutic use[77]. RNAi, with its simplicity of design and specificity, is being investigated for its potential for cancer therapy. RNAi is advantageous in this case, in the sense that it can be used to target a large number of genes involved in different pathways. Genes involved in cancer can be classified into oncogenes, tumor suppressor genes, and tumor promoting genes (growth and angiogenesis) among others. RNAi can be used to silence oncogenes, tumor promoting genes and/or genes that negatively regulate tumor suppressor genes. Cancer-specific genes that are mutated are ideal targets for siRNA therapy as they can be efficiently targeted without affecting the wild type form of the gene[77]. *In vitro* studies using siRNAs directed toward mutated cancer-specific genes have shown extreme specificity towards the mutated form of the gene, whereas silencing of the wild type did not occur[78]. Several studies demonstrated the potential success of RNAi in cancer therapy. *In vitro* study targeting mutated oncogene K-Ras, its expression level was strongly inhibited, *vs* no inhibition of the wild-type. Upon injection of siRNA treated cells into nude mice, tumor formation was dramatically inhibited[77]. Another study targeted the epidermal growth factor receptor; epidermal growth factor receptor (EGFR), which confers an oncogenic activity when mutated leading to promotion of proliferation and survival of the cancerous cell. *In vitro* targeting of EGFR displayed as with K-Ras inhibited expression of mutated EGFR while the wild rapid and massive apoptosis[79]. Another potential target for cancer therapy by RNAi is P-glycoprotein; the product of the multidrug resistance gene.

miRNAs biomarker: miRNAs are regulatory factors that function to repress the transcription of mRNA. Because each miR contains a seed sequence that is complementary to the UTR region of up to around 50 mRNA, the biological impact from the modulation of just a single miR can be significant. Expression profiles are deregulated in cells undergoing pathophysiologic stress suggesting potential as markers of disease states. Based on numerous favorable characteristics of miRNAs as biomarkers as miRNAs are short, protein bound, highly stable in the circulation, and often travel encapsulated in micro vesicles have revealed their potential as diagnostic, prognostic, and treatment response biomarkers.

Several miRNA databases as miRBASE[80], biological databases as those of National Center for Biotechnology Information, and others, ontologies as Gene Ontology, and pathway networks allow investigators to augment and validate relations between miRNA and other information on cellular locations and molecular processes, as well as pathways they contribute to[81,82].

In particular, genetic and epigenetic changes in cells and high frequency of methylated genes in tumors

lead to adenocarcinoma and may serve as a promising marker in the detection of cancer DNA[83,84]. Identification of a panel of biomarker alterations can give us a recognizable pattern of molecular alterations in the HCC which can serve as a "signature" specific for each tumor.

Advanced methods used in identifying biomarkers related to HCC

Numerous recent technologies such as next-generation sequencing (NGS)[85] and microarray technologies[86,87] have adopted in searching for different biomarkers emerged in era of "omics"[88,89]. The progress in high-throughput technologies used in ease way to examine a whole tumor genome (genomics, transcriptomics, proteomics) feature important advances in understanding of the underlying sophisticated pathomechanism for carcinogenesis and metastasis of HCC leading to discover of promising biomarkers with clinical potential. Involving loss of heterogeneity, copy number variations, single nucleotide polymorphism aneuploidy)[90,91], transcriptome[92,93], proteome[94,95], epigenome[83,84], metabolome[96,97], and miRNA profile[98].

The use of genomics and bioinformatics techniques are inevitable for the generation and analysis of comprehensive datasets from patient samples, targeting the detection of hundreds thousands of genetic entities. They have facilitated the investigation of biological entities associated with the progression of tumors - array comparative genomic hybridization (aCGH) array platform have been applied to HCC samples to better deep understand the role of DNA genomic aberrations. Different microarrays companies began by Affymetrix Inc. then applied by Illumina Inc have developed similar approaches containing SNP probes. Numerous studies have used either CGH or aCGH techniques to investigate chromosomal alterations associated with HCC[40]. These array assays based on identification of critical regions commonly exhibit either increased or deletion dosage of gene, leading to alterations in DNA CNVs, aberrations or abnormal LOH blocks in different malignancies, involving liver cancer[99,100].

HUMAN LIVER CANCER PCR ARRAY

Liver cancer PCR Array profiles the expression of many important genes included in the development of HCC. Since numerous microarray studies have identified many deregulated genes, which are important for cellular signaling and other normal biological processes. RT Profiler PCR Array System directed at these genes may yield insights into the molecular mechanisms underlying liver carcinogenesis. This array includes genes commonly up- and down-regulated in HCC, genes involved of signal transduction pathways, and also genes involved in other deregulated biological pathways such as cell cycle, epithelial to mesenchymal transition, inflammation and apoptosis.

Next-generation sequencing

Next-generation sequencing as Roche 454 and Illumina has been recently introduced to enable massive parallel measurement of mRNA and miRNA expression[101]. NGS technology, once reserved for the largest and busiest of research centers, is now attainable to enterprises of all sizes discovering new knowledge on cancer, microbiology, agriculture, genetic disease, reproductive health, and forensics, and other emerging areas. Cancer Sequencing output data are of great important helping in shed light promising newer cancer diagnostics and making data potential of clinical use[102].

Emerging therapeutic strategies for HCC

HCV related HCC is a big health problem in our country Egypt due to the high prevalence of HCV infection among Egyptians. The national committee for viral hepatitis control has started emerging antiviral therapies known as direct acting antivirals. These drugs showed promising increase in the sustained virological response. But what about the role of these drugs on guarding against HCC development?

It is thought that it is early to have a conclusive answer about this question because these drugs act only on the HCV not on liver cells. Moreover, most of the candidate patients for use of these drugs have different grades of liver fibrosis. Can antivirals induce regression of liver fibrosis or even cause stasis of this liver inflammation? The answer for this question needs to follow-up these patients for 20 years at least to detect natural course of liver fibrosis among these patients after control the HCV. Gene therapy for liver cancer means transfer of genetic material to malignant cells, initiating therapeutic effect. This type of emerging therapy may complement or substitute the conventional treatment for HCC. It is important to minimize the transfer of genetic materials to nonmalignant tissues of the liver especially that HCC usually develops on top of cirrhotic liver, thus transfer of genetic material to adjacent non-malignant tissues will accelerate the deterioration of liver functions[103,104].

Inside the target cells, gene expression could be regulated by tumor specific promoters (transcriptional) as surviving or AFP promoters or by targeting messenger RNA of the therapeutic gene (post-transcriptional) for destruction by micro-RNAs. Therapeutic genes used cause cell death through cytotoxicity; inhibition of oncogenic pathway function or stimulation of antitumor immune response[104].

Viral gene therapy procedures aiming to deliver RNAi have shown promising response in animal models of liver cancer through aiming oncogenic pathways involving p28GANK[105], survivin[106,107], VEGF[108] and URG11[109,110].

Other emerging tool of gene therapy for HCC are Replicating virus vectors. Using of specific retroviral replicating vector (RRV) could inhibit the growth of HCC tumor and generate suicide gene therapy effectively with no detectable RRV signal in extratumoral tissues. The resulted tumor-specific suicide-gene-encoding RRV may achieve the engagement application of retroviral gene therapy for HCC cancer[111].

As discussed above, the possible use of miRNA expression in liver cancer as early detectors, miRNAs may themselves be used as therapeutic agents. Different studies clarify the role of miRNAs in HCC tumorigenesis. It was found that miR-26a which is involved in inducing arrest of cell cycle and known to be down-regulated in both human and mouse malignancies, including HCC. In a mouse model of liver cancer, miRNA expression from an AAV vector led to suppression of malignant cell growth and stimulates tumor-specific apoptosis[112].

Recuperation of expression of miRNA 223[113], miRNA 122, lead to decreased metastasis and angiogenesis[114]. Restoration of miRNA101 expression sensitized cells to killing by conventional chemotherapy[115,116]. Silencing of up regulated miRNAs in HCC may lead to disruption of pathways important to tumor survival and development. In this core, it was found that silencing of HCC through specific miR-21 and miR-221 each resulted in reduction of viable and tumor of HCC cells[117,118].

CONCLUSION

There is increasing evidence for HCC tumorigenesis in patients with HCV involves accumulation of genetic alterations. Underlying pathogenic mechanisms of HCC is complex and heterogeneous disease with multiple and variable of risk factors. Thus, signatures of a combination of non-invasive and cost-effective biomarkers may be more valuable for the diagnosis, staging, and prognosis of HCC. Multiple factors contribute in liver carcinogenesis during HCV infection, these factors may act in parallel to each other or they may act in intersecting lines, each in his role. Altogether, these players represent a big challenge in front of conventional therapeutic modalities for HCC. So we think that it's time to try to discover the underlying mechanism for hepatocarcinogenesis to pave the way for development of tailored therapy for those patients changing the basic researches into applied researches. More efforts must be paid by hepatologists especially in countries with high prevalence of HCV infection to identify the underlying genetic mechanisms for liver carcinogenesis among these patients. Cooperation between scientists all over the world with use of recent technology and bioinformatics will build up a strong network for diagnostic markers for HCC and help in early detection of this malignant disease. This network will act as a platform for development of tailored therapy for HCV related HCC.

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Case Control Study

Frontal assessment battery: A tool for screening minimal hepatic encephalopathy?

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Abstract

AIM

To apply the Frontal Assessment Battery to cirrhotic patients with or without overt hepatic encephalopathy (OHE) and controls.

METHODS

The frontal assessment battery (FAB) was applied to 87 patients with liver cirrhosis (16 with and 71 without OHE) and 40 control subjects without cirrhosis treated at the alcohol and liver outpatient clinics and the gastroenterology ward of the Cassiano Antônio de Moraes University Hospital (Hospital Universitário Cassiano Antônio de Moraes - HUCAM), Espírito Santo, Brazil.

RESULTS

The average FAB score was lower for the cirrhotic than for the non-cirrhotic patients (10.6 ± 3.67 *vs* 12.25 ± 2.72, $P = 0.015$). The FAB score was lower for the cirrhotic patients with OHE than for the patients without OHE (8.25 ± 4.55 *vs* 11.14 ± 3.25, $P = 0.027$). The total FAB score was lower for the cirrhotic patients without OHE than for the non-cirrhotic patients, although this difference was not significant (11.14 ± 3.25 *vs* 12.25 ± 2.72, $P = 0.067$). Nevertheless, the difference in the scores on the subtest that assessed the ability to inhibit a response previously conditioned to a stimulus was significant (1.72 ± 0.93 *vs* 2.2 ± 0.85, $P = 0.011$).

CONCLUSION

The present study indicates that the FAB is a promising

tool for outpatient minimal HE screening and the assessment of HE severity.

Key words: Executive functions; Frontal lobe; Hepatic encephalopathy; Minimal hepatic encephalopathy; Liver cirrhosis; Frontal assessment battery

Core tip: The diagnosis of hepatic encephalopathy is based on the West Haven classification. Minimal hepatic encephalopathy is defined by cognitive changes in patients with liver cirrhosis or portosystemic shunting without changes in their physical examination. The diagnosis is performed by neurophysiological and/or neuropsychological tests that are difficult to apply and are expensive. The frontal assessment battery (FAB), which is quick and easy to apply, can be used by the clinician. In the present study, the FAB score was lower in cirrhotic patients, especially those with hepatic encephalopathy. The FAB is a promising test for minimal hepatic encephalopathy screening at the bedside and in outpatient clinics.

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INTRODUCTION

Hepatic encephalopathy (HE) comprises a heterogeneous group of neuropsychiatric disorders that occur in patients with liver cirrhosis (LC) or portosystemic shunting in the absence of other known brain diseases[1]. The traditional HE classification comprises four grades based on the West Haven criteria for the semi-quantitative grading of mental status[2,3]. Overt HE (OHE) is characterized by the presence of clinical manifestations that are easily recognizable in clinical interviews and physical examinations and corresponds to grades 2 to 4. Subclinical HE includes forms of the disease that are not easily recognizable in the clinical interview and physical examination, such as West Haven grade 1 and minimal HE (MHE), in which the cognitive deficits can only be detected using specialized tests[4,5]. The cognitive deficits caused by MHE have negative impacts on the social and occupational lives of the patients and thus impair their quality of life[2] and are associated with a higher risk of accidents[6]. Because MHE cannot be recognized during a physical examination, its diagnosis is established through the application of tests to apparently normal individuals who exhibit risk factors for the development of this condition[7].

Traditionally, the MHE diagnosis is established based on neurophysiological and/or neuropsychological tests[4]. The majority of these tests are extensive and require a significant amount of time to perform; additionally, they are difficult to interpret and require the expertise of neuropsychiatry professionals[8]. The frontal assessment battery (FAB) developed by Dubois *et al*[9] is a quick tool; its application requires approximately 10 min. It is well accepted by patients and is useful for identifying the presence and assessing the severity of dysexecutive syndromes that affect cognition and motor behavior[10]. The FAB comprises six subtests (conceptualization, lexical fluency, motor series, conflicting instructions, inhibitory control, and automatic behavior)[9,11]. Originally, the FAB was used to assess patients with Parkinson's disease who exhibited abnormalities on the Wisconsin Card Sorting Test, Trail Making Test and verbal fluency tests[9,12] and patients with abnormal perfusion of the frontal lobe on imaging tests[13-17]. These studies suggest that the FAB evaluates executive functions of the frontal lobe. Additionally, it has proven useful for distinguishing between Alzheimer's disease (AD) and frontotemporal dementia (FTD)[18], detecting subclinical dysexecutive alterations in alcoholic subjects, formulating differentiated therapeutic strategies for the management of alcoholic patients on an individual basis[19], and correlating the use of crack cocaine with a decline in the frontal executive functions as a function of the duration of drug use[20]. The present study represents the first application of the FAB in patients with chronic liver disease. The results are compared between individuals with OHE and subclinical HE and the controls.

The aim of the present study was to determine whether the FAB could detect differences between patients with LC and the controls and between cirrhotic patients with and without OHE and to investigate whether this tool might be indicated for outpatient screening for MHE.

MATERIALS AND METHODS

Patients

The present study assessed 127 individuals treated at the gastroenterology ward and liver and alcohol outpatient clinics of Cassiano Antônio de Moraes University Hospital, Federal University of Espírito Santo (Hospital Universitário Cassiano Antônio de Moraes, da Universidade Federal do Espírito Santo), Espírito Santo, Brazil. A total of 87 patients had LC, including 16 with OHE and 71 without HE. The remaining 40 patients were defined as controls and were matched according to gender and the cirrhosis etiology (alcoholism, hepatitis B, or hepatitis C). All LC patients were assessed and classified based on the Child-Pugh classification for the severity of liver disease. Individuals with clinical manifestations of psychiatric diseases, those who had consumed alcohol in the past 15 d, and those under 18 years of age were excluded from the study.

LC diagnosis

The LC diagnosis was established based on the com-

Table 1 Frontal assessment battery

Frontal assessment battery
(1) Similarities
In what way are they alike?
(a) A banana and an orange
(b) A table and a chair
(c) A tulip, a rose, and a daisy
3 correct: 3
2 correct: 2
1 correct: 1
None correct: 0
(2) Lexical fluency
Say as many words as you can begin with the letter "S", except for proper nouns
(3) Motor series
Fist, palm, edge (first together, then alone)
6 correct consecutive series alone: 3
At least 3 correct consecutive series alone: 2
3 correct consecutive series with the examiner: 1
Cannot perform 3 correct consecutive series even with the examiner: 0
(4) Conflicting instructions
Tap twice when I tap once
Tap once when I tap twice
No errors: 3
1-2 errors: 2
More than 2 errors: 1
Patient imitates the taps of the examiner at least 4 consecutive times: 0
(5) Go/No Go
Tap once when I tap once
Do not tap when I tap twice
No errors: 3
1-2 errors: 2
More than 2 errors: 1
Patient imitates the taps of the examiner at least 4 consecutive times: 0
(6) Behavior
Do not touch my hands
The patient's hands should be on his/her knees with the palm up. Without saying anything, the examiner brings his/her own hands close to the patient's. If the patient touches the examiner's hands, the examiner says: Now, do not touch my hands. Then, a new attempt begins
Patient does not touch the examiners hands: 3
Patient hesitates and asks what he/she has to do: 2
Patient touches the examiner's hands without hesitation: 1
Patient touches the examiner's hands even after he/she was told not to do so: 0

bination of clinical criteria and the results of imaging and/or histopathological tests.

HE diagnosis

Patients with LC and neuropsychiatric disorders detected during the clinical interviews and physical examinations in the absence of other known brain diseases were diagnosed with OHE[1]. The following clinical manifestations were considered: Behavioral alterations, sleep disorders, irritability, and depression. The psychomotor abnormalities included asterixis, bradykinesia, tremors, and rigidity. Additionally, mental confusion and acute temporal-spatial disorientation were considered[21].

FAB

The FAB was applied to patients individually by the same examiner using only a paper and pencil; the tests were applied to inpatients at the bedside and at the outpatient clinics. The FAB is described in Table 1.

To compare the FAB scores to dichotomous categorical variables, the scores were categorized as high (> 11) or low (⩽ 11) based on the median scores of the LC patients.

Ethics

The present study was approved by the ethics committee of the Center of Health Sciences, Federal University of Espírito Santo (Universidade Federal do Espírito Santo), which is associated with the National Commission of Research Ethics (Comissão Nacional de Ética em Pesquisa - CONEP). After receiving a detailed explanation of the study, the participants signed an informed consent form approved by the institutional medical ethics committee.

Statistical analysis

The statistical analysis was performed with the Epi Info 6.04 e BioEstat 5.3 software. The data were subjected to a descriptive analysis, including the frequency distribution (in the case of qualitative variables), are expressed as absolute numbers (*n*) and percentages (%), and the means and standard deviations (SD) were calculated. The means of data with a normal distribution were compared using Student's *t*-test. Categorical variables were subjected to a cross-tabulation analysis with a χ^2 test. Fisher's exact test was used to compare two variables when the expected frequency according to the null hypothesis was less than five, and the maximum likelihood ratio was used when the exposure variable comprised more than two categories.

The non-parametric Mann-Whitney test was used for continuous variables with a non-normal distribution. Associations with a value of $P < 0.05$ were considered statistically significant.

RESULTS

Patient characteristics

The demographic characteristics of the sample are described in Table 2. The predominant cause of cirrhosis was alcoholism (65.5%). The cirrhosis etiology and the underlying diseases of the control subjects are described in Table 3. No significant differences were detected in the FAB scores related to the liver cirrhosis etiology.

Among the 87 cirrhotic patients, 33 were classified as Child-Pugh class A, 36 were classified as class B, and 17 were classified as class C. Most of the cirrhotic patients with OHE were classified as Child-Pugh class C (56% *vs* 11% of the cirrhotic patients without OHE, $P < 0.001$).

Figure 1 shows that no difference was observed in the FAB scores as a function of the cause of disease when the cirrhotic patients and controls were compared.

The FAB score was lower (mean 10.6, SD ± 3, maximum score 18) for the cirrhotic patients than for the controls (mean 12.5, SD ± 2.72, $P = 0.015$). For the cirrhotic patients with OHE, the average score was

Table 2 Characterization of the cirrhotic and non-cirrhotic patients *n* (%)

Variables	Cirrhotic (*n* = 87)	Non-cirrhotic (*n* = 40)	*P*-value
Gender			0.814
Male	68 (78)	32 (80)	
Female	19 (22)	8 (20)	
Age range			0.001
50 yr or older	69 (79)	20 (50)	
Under 50 yr	18 (21)	20 (50)	
Formal schooling			0.577
None	5 (6)	3 (7.5)	
1 to 3 yr	15 (17)	3 (7.5)	
4 to 7 yr	33 (38)	18 (45)	
8 to 11 yr	28 (32)	12 (30)	
12 or more years	6 (7)	4 (10)	

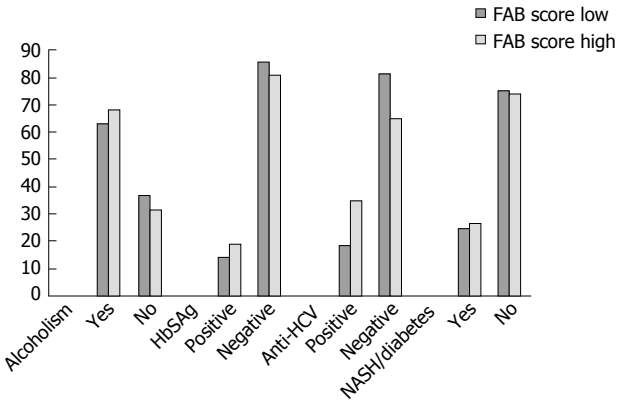

Figure 1 Percent distribution of the frontal assessment battery score, categorized as high or low, of the investigated patients (cases and controls) compared according to the cause of liver cirrhosis (Fisher's exact test). FAB: Frontal assessment battery; HbSAg: Hepatitis B virus surface antigen; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis.

8.25 (SD ± 4.55) and the median score was 7.5. In comparison, the scores of the cirrhotic patients without HE were higher (mean 11.4, SD ± 3.25, median 11, P = 0.027). The FAB score was lower for the cirrhotic patients without OHE than for the controls, although the difference was not significant (P = 0.067) (Figure 2).

The poorest scores in the cirrhotic group corresponded to the inhibitory control subtest (GO/NO-GO) (mean 1.61, SD ± 0.98) compared with the controls (mean 2.2, SD ± 0.85, P = 0.02). The GO/NO-GO scores were lower for the cirrhotic group without OHE (mean 1.72; SD ± 0.93) than for the controls (mean 2.2, SD ± 0.85, P = 0.011). The GO/NO-GO scores were also lower for the cirrhotic group with OHE (mean 1.13, SD ± 1.09) than for the cirrhotic group without OHE (mean 1.72, SD ± 0.93, P = 0.02), as shown in Figure 3. In this subtest, the individuals were required to inhibit a learned behavior (clap once when the examiner claps twice) and then perform a different task (do not clap when the examiner claps twice). No significant differences were detected in any of the other subtests in the comparison between the cirrhotic patients without OHE and the control group (Figure 4).

Of the 16 cirrhotic patients with OHE, 12 exhibited

Table 3 Comparison of causes of cirrhosis and the underlying diseases of non-cirrhotic patients *n* (%)

Variables	Cirrhotic (*n* = 87)	Non-cirrhotic (*n* = 40)	*P*-value
Alcoholism			0.434
Yes	57 (65.5)	29 (72.5)	
No	30 (34.5)	11 (27.5)	
Hepatitis B surface antigen			0.864
Positive	14 (16)	7 (17.5)	
Negative	72 (84)	33 (82.5)	
Anti-hepatitis C virus antibodies			0.316
Positive	22 (25.6)	7 (17.5)	
Negative	64 (74)	33 (82.5)	
Non-alcoholic steatohepatitis/ diabetes			0.007
Yes	22 (25)	2 (5)	
No	65 (75)	38 (95)	

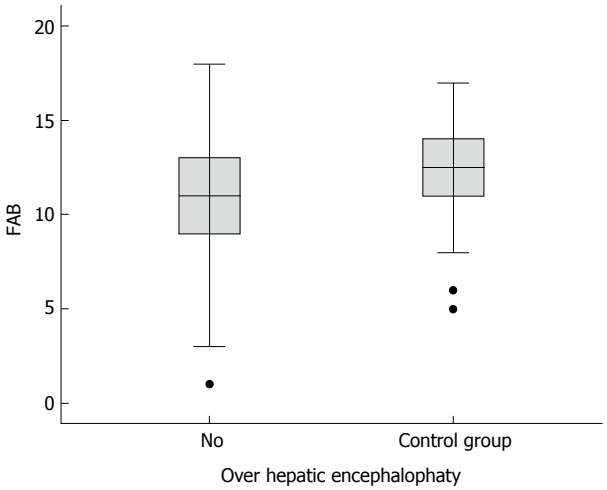

Figure 2 Median (interquartile range) total scores on the frontal assessment battery for cirrhotic patients with overt hepatic encephalopathy (*n* = 16) *vs* cirrhotic patients without overt hepatic encephalopathy (*n* = 71) (Student's *t*-test) and the control group (non-cirrhotic patients, *n* = 40) *vs* the case group (cirrhotic patients with and without overt hepatic encephalopathy, *n* = 87) (Mann-Whitney test). FAB: Frontal assessment battery.

low FAB scores (12/16, 75%). Low FAB scores were exhibited by 37/71 (52%) cirrhotic patients without HE and 17/40 (43%) controls. The linear association test detected a significant difference among the groups (P = 0.038).

DISCUSSION

Several studies have shown that MHE causes abnormalities in the attention, social interactions, behavior, and quality of sleep of patients, with consequent impairment of the performance of the activities of daily living. Additionally, interference with more complex activities, such as driving ability or planning a trip, impairs the quality of life of patients and may increase the risk of accidents involving themselves and others[2].

The investigation of patients with cirrhosis by the West Haven test is not sufficient to identify subclinical forms of encephalopathy[22]. Traditionally, the MHE

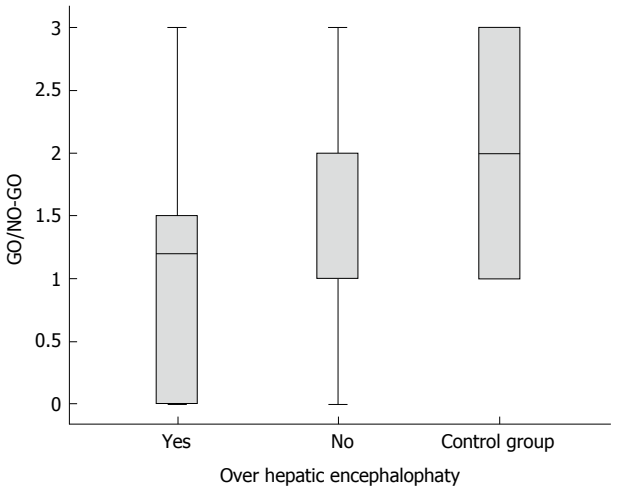

Figure 3 Median (interquartile range) total scores on the inhibitory control subtest (GO/NO-GO) for cirrhotic patients with overt hepatic encephalopathy (*n* = 16) *vs* cirrhotic patients without overt hepatic encephalopathy (*n* = 71) (Student's *t*-test) and the control group (non-cirrhotic patients, *n* = 40) *vs* the case group (cirrhotic patients with and without overt hepatic encephalopathy, *n* = 87) (Mann-Whitney test).

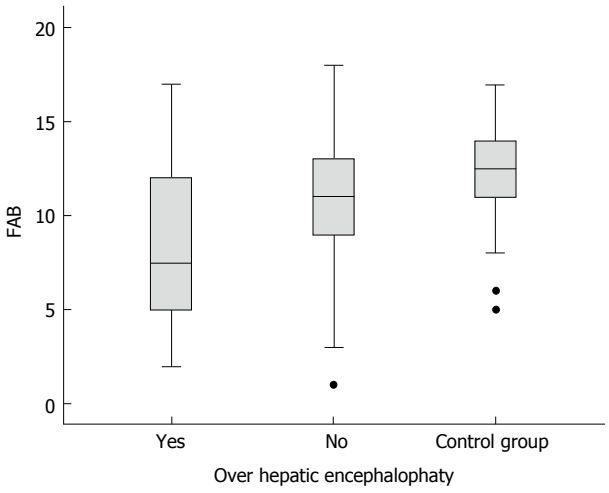

Figure 4 Median (interquartile range) total scores on the frontal assessment battery for cirrhotic patients without overt hepatic encephalopathy *vs* the control group (non-cirrhotic patients) (Mann-Whitney test). FAB: Frontal assessment battery.

diagnosis is established based on the detection of neurological dysfunctions in neurophysiological and/or neuropsychological tests[4]. However, these tests are rather long, time consuming, and difficult to interpret[8]. Dhiman *et al*[3] suggested that the Psychometric Hepatic Encephalopathy Score, which is a battery of neuropsychological tests that can detect abnormalities such as alterations of motor function, visuospatial orientation, visual perception, visual construction, attention, concentration, and (with somewhat lower efficacy) memory disorders, should be considered the gold standard for the assessment of MHE, whereas computer-based tests such as Critical Flicker Frequency and Inhibitory Control Test should be used for screening. However, neurophysiological tests are difficult to apply in an outpatient setting because they require modern facilities and equipment[2].

A study was conducted in which the Mini-Mental State Examination was applied to patients with LC and OHE, MHE, or without HE to establish whether this test might be used as a screening method for MHE and HE West Haven grades 1 and 2. However, a significant difference was not found between the scores of patients with MHE and those without HE. Moreover, alteration of the mental status was only detected in patients with OHE West Haven grade 3, which could be clinically detected without additional assessment methods[21].

Citro *et al*[22] conducted a study that applied the Trail Making Test (a simple inexpensive test) in a recent series evidenced a poor psychometric performance in more than half of the patients who were free of manifest encephalopathy. The authors also observed that subclinical hepatic encephalopathy was mostly present in patients with HCV-related cirrhosis. In the present study, there was no difference in FAB scores related to the cirrhosis etiology.

The present study used the FAB described by Dubois *et al*[9] as a useful and practical tool to establish the presence and severity of dysexecutive syndromes affecting cognition and motor behavior[9] and to assess patients with and without LC.

Some studies have indicated that the FAB evaluates the executive functions of the frontal lobe. In clinical practice, the FAB has also been used to distinguish between AD and FTD at the bedside, even in the earliest stages of disease[23]. One case-control study compared a group of 170 alcoholic subjects to a group of 40 non-alcoholic controls to assess frontal functions in different categories of alcoholism according to the Lesch typology. The use of the FAB as an assessment instrument allowed the detection of subclinical dysexecutive abnormalities among the alcoholic subjects. These data might serve to formulate differentiated therapeutic strategies for the management of alcoholic patients on an individual basis[20].

The FAB was also used as a tool in a descriptive cross-sectional case series study of 72 crack cocaine users in which their patterns of drug use, global cognition, and frontal executive functions were assessed. The results indicated a decline in executive functions associated with the duration of drug use, especially for the functions investigated by the Automatic behavior subtest[20].

No reports exist in the literature of studies investigating the frontal executive functions in cirrhotic patients. Therefore, no reference values for normality existed to compare with the results of the cirrhotic and non-cirrhotic patients. In the present case-control study, the FAB was applied to cirrhotic and non-cirrhotic patients. The performance of the former group was poorer, suggesting a considerable difference in the performance on the FAB between these two patient groups. The lower scores exhibited by the cirrhotic patients might be attributed to the presence of MHE, which is a subclinical condition that affects less than 15% of cirrhotic Child-Pugh class A patients and approximately

50% of patients classified as Child-Pugh class B or C[10]. Among patients with advanced liver disease according to the Child-Pugh classification, the scores of patients classified as Child-Pugh class C were not significantly different from the overall group of cirrhotic patients who exhibited the lowest FAB scores. However, the cirrhotic patients classified as Child-Pugh class C exhibited poorer performance in the GO/NO-GO and Automatic behavior subtests.

Abnormalities in the FAB GO/NO-GO subtest indicate difficulty in inhibiting inappropriate responses due to injury to the ventral part of the frontal lobe[9]. In the present study, the scores exhibited by cirrhotic patients were significantly lower than the scores of the non-cirrhotic patients. This finding might be explained by the executive dysfunction caused by MHE[8].

No other FAB subtest scores (conceptualization, verbal fluency, motor series, conflicting instructions, and automatic behavior) were significantly different between the cirrhotic and non-cirrhotic patients.

Detecting minimal hepatic encephalopathy in patients with cirrhosis may help improve their quality of life[22]. The FAB score was lower for patients with OHE than for cirrhotic patients without OHE. This finding indicates that the FAB can detect the psychomotor abnormalities characteristic of OHE[1].

Regarding the FAB subtests, patients with OHE exhibited lower scores on the Motor series subtest, which indicated injury to the frontal lobe that impaired temporal organization and the maintenance and execution of successive actions. Additionally, this group of patients exhibited lower scores on the GO/NO-GO subtest, in which abnormalities indicated difficulty inhibiting inappropriate responses. These findings show that the FAB detects HE-related cognitive dysfunctions in the early subclinical stage of disease[2] *via* the GO/NO-GO subtest as well as the psychomotor abnormalities characteristic of OHE, which are recognizable in the physical examination[3] *via* the Motor series subtest.

Although the FAB score exhibited by the cirrhotic patients without MHE was lower than the average score of the controls, the difference was not significant. Further studies with larger sample sizes are needed to thoroughly investigate this difference. The score on the GO/NO-GO subtest was significantly lower for the cirrhotic patients without OHE than for the non-cirrhotic patients. We may infer that this difference is due to the presence of MHE-related executive dysfunctions in some cirrhotic patients who do not have clinical OHE manifestations.

Currently, no studies have applied the FAB to cirrhotic patients with or without complications such as HE to establish the cutoff point for defining normality. However, the differences in the FAB scores between the cirrhotic and non-cirrhotic patients and between patients with and without HE in the present study demonstrate its value. Further studies are needed to determine whether the FAB could be used as a screening tool for all grades of OHE severity and particularly as a helpful tool for the assessment of West Haven grade 1 and MHE in clinical practice[1].

In conclusion, the FAB is a promising tool with an easy and quick application that may be used by trained general practitioners in both the outpatient setting and at the bedside as a screening method for West Haven grade 1 HE and MHE. Further studies are needed to validate this tool and compare it to other neuropsychometric batteries currently used to detect MHE.

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COMMENTS

Background

The usual diagnostic minimal hepatic encephalopathy (MHE) tests are time consuming and require the participation of specialized professionals. The delay in diagnosis has a negative impact on the quality of life of patients and leads to a higher risk of progression to overt HE. The availability of an easily applicable screening test for MHE, such as the frontal assessment battery (FAB), allows early detection of MHE and its treatment.

Research frontiers

The FAB is a battery of tests with easy and quick application that was originally used for the evaluation of patients with dysexecutive syndromes of the frontal lobe, such as Parkinson's disease, chronic alcohol abusers and crack users. The FAB can also be useful in differentiating the frontotemporal dementia of Alzheimer's disease. Therefore, the FAB would be a useful tool for evaluating the presence and severity of dysexecutive syndromes that affect cognition and motor behavior in liver cirrhosis patients.

Innovations and breakthroughs

Previous articles in the literature unsuccessfully proposed simple and inexpensive screening tests for MHE, such as the Mini Mental and Trail Making Test. However, the application of the FAB for the evaluation of MHE and HE in patients with liver cirrhosis is unique. Their study suggests that the FAB should be considered a promising tool for screening MHE and HE by the clinician.

Applications

The FAB is a promising tool for screening HE and MHE in patients both at the bedside and in outpatient clinics.

Peer-review

It is a good paper.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective study of the associations between hepatitis C virus infection and metabolic factors

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Abstract

AIM

To evaluate the bidirectional association between metabolic syndrome (MS) components and antiviral treatment response for chronic hepatitis C virus (HCV) infection.

METHODS

This retrospective cohort study included 119 HCV + patients treated with pegylated-interferon-α and ribavirin. Metabolic characteristics and laboratory data were collected from medical records. Differences in baseline clinical and demographic risk factors between responders and non-responders were assessed using independent samples *t*-tests or χ^2 tests. The effects of sustained viral response (SVR) to antiviral treatment on *de novo* impairments in MS components, including impaired fasting glucose (IFG) and type 2 diabetes mellitus (T2DM), were assessed using univariable and multivariable logistic regression analysis, while the effect of MS components on SVR was assessed using univariable logistic regression analysis.

RESULTS

Of the 119 patients, 80 (67%) developed SVR over the

average 54 ± 13 mo follow-up. The cumulative risks for *de novo* T2DM and IFG were 5.07- (95%CI: 1.261-20.4, *P* = 0.022) and 3.87-fold higher (95%CI: 1.484-10.15, *P* = 0.006), respectively for non-responders than responders, when adjusted for the baseline risk factors age, sex, HCV genotype, high viral load, and steatosis. Post-treatment triglyceride levels were significantly lower in non-responders than in responders (OR = 0.27; 95%CI: 0.069-0.962, *P* = 0.044). Age and HCV genotype 3 were significantly different between responders and non-responders, and MS components were not significantly associated with SVR. Steatosis tended to attenuate SVR (OR = 0.596; 95%CI: 0.331-1.073, *P* = 0.08).

CONCLUSION

SVR was associated with lower *de novo* T2DM and IFG incidence and higher triglyceride levels. Patients infected with HCV should undergo T2DM screening and antidiabetic treatment.

Key words: Hepatitis C virus; Type 2 diabetes mellitus; Antiviral therapy; Sustained viral response; Metabolic syndrome; Hepatic steatosis; Peg interferon alpha; Ribavirin; Direct acting antiviral agents

Core tip: Hepatitis C virus (HCV) is associated with a unique metabolic syndrome (MS) type: Insulin resistance with type 2 diabetes mellitus (T2DM), hypocholesterolemia, and liver steatosis. We retrospectively investigated the association between MS components and HCV infection, including antiviral therapy response, for 119 patients infected with HCV treated with interferon alpha and ribavirin. After long-term follow-up, *de novo* T2DM incidence significantly decreased, and triglyceride levels significantly increased in treatment responders. Only steatosis tended to affect treatment response. The association between HCV and lipid metabolic pathways may be important even with new direct antiviral agents. Patients infected with HCV should be screened for T2DM.

Yair-Sabag S, Nussinson E, Ben-Assuli O, Shibli F, Shahbari A, Zelber-Sagi S. Retrospective study of the associations between hepatitis C virus infection and metabolic factors. *World J Hepatol* 2016; 8(30): 1269-1278 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i30/1269.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i30.1269

INTRODUCTION

A mutual association between hepatitis C virus (HCV) infection and host metabolism has been demonstrated in several studies. HCV depends on host lipids for entry into the hepatocytes and for its replication; in return, HCV also affects the metabolism of host lipids[1-3].

HCV causes insulin resistance, hepatic steatosis, type 2 diabetes mellitus (T2DM), and low serum cholesterol and triglyceride (TG) levels. Insulin resistance contributes to HCV-related disruption of glucose and lipid metabolism[4], and it is a key factor in metabolic syndrome (MS). In addition, HCV infection might lead to hepatic steatosis *via* several pathways.

Hepatic steatosis might aggravate MS directly by causing further insulin resistance[5] or indirectly because of resultant hepatic fibrosis[6] or cirrhosis[4,7,8]. After HCV infection, cholesterol and TG levels decrease, creating a different lipid profile from that for MS[9]. However, T2DM might be twice as prevalent in patients infected with HCV compared to the general population[5]. HCV has been associated with a unique type of MS called hepatitis C-associated dysmetabolic syndrome (HCADS), which includes liver steatosis, insulin resistance, and hypocholesterolemia[5,10]. Reversal of hypocholesterolemia and steatosis after achieving sustained viral response (SVR) with antiviral therapy has been observed in several studies[11-13].

Therefore, although MS is not clearly associated with HCV, there is an association between HCV and some MS components. HCV-induced fatty liver and insulin resistance leads to T2DM; with the additional presence of MS, HCV replication is accelerated by activation of hepatocyte transcription factors, leading to increased lipogenesis and the provision of lipids for HCV replication[5,9,10]. Furthermore, in patients with MS, immune responses to HCV can be attenuated by leptin resistance or other changes in adipokine secretion[5]. Thus, MS might interfere with SVR after treatment[11,14-17].

Previous studies showed that HCV eradication decreases the risk of *de novo* glucose abnormalities and insulin resistance. On the other hand, some studies reported neither an association between metabolic syndrome and HCV infection[18] nor reduced incidence of *de novo* glucose abnormalities in responders to treatment with interferon alpha and ribavirin[19].

Our study aimed to assess the association between MS components and HCV infection based on the response to the therapy as well as to evaluate the influence of MS components on the response to antiviral therapy in a younger cohort of HCV-infected patients with a long term follow-up.

MATERIALS AND METHODS

During 2004-2008, 119 patients diagnosed with chronic HCV infection, based on positive HCV RNA findings on polymerase chain reaction (PCR), were treated with combination pegylated-interferon α (Peg-IFNα) and ribavirin in the department of gastroenterology at Emek Medical Center in Afula, Israel. All patients were eligible for the Peg-IFNα and ribavirin treatment, which consisted of 180 μg Peg-IFNα administered subcutaneously once a week and 800-1200 mg ribavirin administered orally

daily. Treatment lasted 24 wk for patients with genotypes 2/3 and 48 wk for those with genotypes 1/4.

Patients with hepatocellular carcinoma (HCC), human immunodeficiency virus infection, other serious conditions, or evidence of drug abuse or excessive alcohol consumption during the year preceding the enrollment were excluded.

Metabolic and demographic characteristics

Patients were evaluated before treatment, 6 mo after treatment, and every year after the end of treatment until 2011. Age, body mass index (BMI), serum fasting blood glucose, TG, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, aspartate transaminase, alanine transaminase, viral load, blood pressure, liver biopsy results, and liver ultrasonography were obtained from medical records and were not available in some cases. To reduce data bias, all data were extracted from several independent sources, including patient hospital files, electronic files from the family physician, and laboratory results every year after the end of treatment. The study was performed after obtaining local ethics committee approval.

Impaired fasting glucose (IFG) was defined as a serum fasting glucose level > 100 mg/dL, and T2DM was identified based on diagnoses documented in medical records, serum fasting glucose level > 126 mg/dL, or use of antidiabetic drugs. MS was defined using the World Health Organization clinical criteria for MS [20].

Serum anti-HCV antibodies were measured using a 2nd generation immunoassay, and HCV RNA was measured using real time-PCR (RT-PCR; Amplicor HCV test, Roche Diagnostic; detection rate = 50 IU/mL). HCV genotyping was determined using RT-PCR (HCV genotyping, DNA immunoassay).

Most, but not all, patients agreed to and underwent a baseline liver biopsy to determine hepatic inflammation and steatosis. In addition, ultrasonography was performed before treatment and during the follow-up period to determine hepatic steatosis.

Statistical analysis

The statistical methods of this study were reviewed by Shira Zelber-Sagi from the School of Public Health at the University of Haifa and Ofir Ben-Assuli from Ono Academic College.

Continuous variables (MS components) are presented as means ± SD. Statistical analyses were performed using SPSS version 21 (IBM Corp., Armonk, NY, United States), and $P < 0.05$ was considered statistically significant for all analyses.

The response to treatment was the independent variable, and metabolic components were initially evaluated separately as dependent variables using independent samples *t*-tests or Mann-Whitney U tests, when appropriate, for continuous variables (*e.g.,* age and BMI) and Pearson's χ^2 tests and odds ratios (ORs) for categorical variables (*e.g.,* sex and genotype).

To test differences from baseline to the average follow-up duration in continuous variables between the treatment responders and non-responders, independent samples *t*-tests were performed.

The difference in *de novo* occurrence of MS components (0 = normal values; 1 = abnormal values indicating presence of the component) between responders and non-responders was calculated after excluding patients with MS components prior to antiviral treatment. Then, logistic regression analysis of *de novo* occurrence of T2DM and other MS components was conducted at different time intervals using an unadjusted (univariable) logistic regression (model 1) and an adjusted (multivariable) logistic regression (model 2, adjusted for age, sex, BMI, and genotype). Additionally, the cumulative rates of the patients without *de novo* occurrence of IFG, T2DM, and MS were estimated using the Kaplan-Meier method and compared between responders and non-responders using the log-rank test.

The effects of metabolic, demographic, and clinical variables on treatment response were determined using univariable logistic regression analysis.

RESULTS

The mean age of the 119 HCV-positive patients treated with Peg-IFNα and ribavirin (57% men, 43% women) was 41 ± 11.3 years (Table 1). The sample population primarily included immigrants from the Union of Soviet Socialist Republics (77%). The proportions of patients with HCV genotypes 1, 2, 3 and 4 were 66%, 9.2%, 22% and 1.7%, respectively (Table 1). The mean follow-up duration for MS components after treatment was 47.5 ± 13.3 mo.

Regarding MS components, hypertension, T2DM, and IFG were present in 17%, 9.2%, and 27.7% of patients, respectively (Table 1). Serum HDL values were within the lower limit of the normal range, and serum TG levels were within the normal range. Mean BMI was in the overweight range (27 ± 5.4 kg/m^2). Steatosis was present in 36% or 16.5% of patients, as determined with liver biopsy or abdominal ultrasound, respectively (Table 1).

SVR was obtained in 67% (n = 80) of patients. Only baseline age and HCV genotype 3 were significantly different between responders and non-responders (Table 2). Non-responders were significantly older (P = 0.017), and significantly fewer non-responders had HCV genotype 3 (P = 0.005).

In the unadjusted regression analysis of the effect of the baseline metabolic factors on treatment response, metabolic syndrome and metabolic components (except T2DM) negatively affected treatment response (OR = 0.448; OR = 0.597; respectively), though none of them were significantly associated with treatment response (P = 0.847 and P = 0.483; respectively) (Table 3).

Table 1 Baseline characteristics of the hepatitis C virus-infected patients treated with pegylated-interferon α and ribavirin

Variable (normal values)	*n*[1]	
Age (yr)	119	41 ± 11.3
Sex (men %)		57.1
Birth place (%)		
Israel		12.6
Union of Soviet Socialist Republics		77.3
Other (Europe, North America, South Africa, Georgia)		10
Viral load (IU/mL)	114	461.234 ± 251.445
HCV genotype (%)		
1		66.4
2		9.2
3		22.7
4		1.7
BMI (19-25 kg/m^2)	81	27.0 ± 5.4
Systolic BP (15.99 kPa)	108	15.59 ± 2.26
Diastolic BP (10.66 kPa)	108	9.06 ± 1.56
Serum glucose (70-100 mg/dL)	98	96.96 ± 20.5
Cholesterol (100-200 mg/dL)	117	176 ± 49
Triglycerides (30-150 mg/dL)	90	116 ± 65
HDL (40-60 mg/dL)	74	48.8 ± 12
AST (3-32 IU)	119	51 ± 32
ALT (3-33 IU)	119	77 ± 59
T2DM (diagnosis, fasting blood glucose > 126 mg/dL, or use of anti-diabetic drugs) (%)		9.2
IFG or T2DM (fasting blood glucose > 100 mg/dL) (%)		27.7
Steatosis determined using liver biopsy (*n* = 85)[2]		
Without steatosis (%)		36
Mild		37.3
Moderate		25.3
Severe		1.3
Steatosis determined using abdominal ultrasound (*n* = 110; with steatosis) (%)		16.5

Values are reported as mean ± SD or %. [1]Data were not available for some patients due to the retrospective nature of the study; [2]Some of the patients refused to undergo liver biopsy. HCV: Hepatitis C virus; BMI: Body mass index; BP: Blood pressure; HDL: High-density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T2DM: Type 2 diabetes mellitus; IFG: Impaired fasting glucose.

Table 2 Comparison of baseline characteristics between responders and non-responders to treatment with pegylated-interferon α and ribavirin

Variable	Non-responders	Responders	*P* value[1]
Age (yr)	44.62 ± 11.22	39.42 ± 11.14	0.017
BMI (kg/m^2)	26.88 ± 5.11	27.34 ± 5.70	0.717
Genotype 3 (%)	2.7	17.4	0.005
Male sex (%)	20.8	35.6	0.736

The values are reported as mean ± SD or percentages. [1]*P* values determined using independent-samples *t*-tests. BMI: Body mass index.

In the unadjusted regression analysis of the effect of the baseline demographic and clinical variables on treatment response, the ORs for HCV genotype 3 and age were significant (OR = 5.35; 95%CI: 1.48-19.3; *P* = 0.01 and OR = 0.959; 95%CI: 0.926-9.93; *P* = 0.019; respectively), suggesting positive effects of genotype 3 and relatively young age on response to treatment (Table 4). While the rate of hepatic steatosis as determined using abdominal ultrasound (16.5%) was not significant, the rate of hepatic steatosis as determined by liver biopsy (64%) tended to result in a better response (*P* = 0.085) (Table 4).

Table 3 Unadjusted logistic regression analysis of the association between baseline metabolic components and antiviral treatment response (*n* = 115)

Variables	Crude OR	95%CI (*P* value)
BMI > 30 kg/m^2	0.825	0.303-2.243 (0.706)
IFG (> 100 mg/dL)	0.609	0.266-1.393 (0.140)
T2DM (diagnosis, fasting blood glucose > 126 mg/dL, or use of anti-diabetic drugs)	1.094	0.301-3.975 (0.892)
High blood pressure (> 16/10.66 kPa)	0.713	0.269-1.889 (0.495)
High triglycerides	1.075	0.338-2.978 (0.889)
High cholesterol and low HDL levels	0.782	0.367-1.666 (0.523)
Presence of any metabolic syndrome components (high cholesterol levels, hyperlipidemia, high BP, or BMI > 30), without T2DM	0.448	0.551-1.301 (0.847)
Metabolic syndrome	0.597	0.141-2.520 (0.483)

The non-responder group is the reference group. OR: Odds ratio; BMI: Body mass index; IFG: Impaired fasting glucose; T2DM: Type 2 diabetes mellitus; HDL: High-density lipoprotein cholesterol; BP: Blood pressure.

Univariable and multivariable logistic regression analyses of the effect of antiviral therapy response on the *de novo* impaired MS components resulted in significant

Table 4 Univariate analysis of the association between baseline demographic, clinical, and laboratory variables and successful treatment response

Variables	Crude OR	95%CI (*P* value)
Sex (*n* = 115)	0.878	0.412-1.873 (0.737)
Mean age (yr) (*n* = 115)	0.959	0.926-9.93 (0.019)
Birth place (Israel/Union of Soviet Socialist Republics/other) (*n* = 115)	0.839	0.530-1.329 (0.455)
Current smoker (yes/no) (*n* = 105)	1.487	0.762-2.901 (0.245)
Alcohol consumption (none/past) (*n* = 103)	1.133	0.266-4.824 (0.866)
Drug use (none/past user) (*n* = 106)	1.476	0.550-3.961 (0.439)
Genotype 3 (*n* = 115) (genotypes 1, 2, and 4 are grouped as the reference)	5.35	1.48-19.3 (0.010)
Liver steatosis determined by biopsy (yes/no) (*n* = 74)	0.596	0.331-1.079 (0.085)
Liver steatosis determined by ultrasound (yes/no) (*n* = 107)	0.515	0.181-1.462 (0.213)

Data were not available for some patients due to the retrospective nature of the study. OR: Odds ratio.

Table 5 Unadjusted (model 1) and adjusted (model 2) logistic regression analyses of the association between the response to hepatitis C antiviral treatment and the de novo occurrence of metabolic syndrome components

Variable	Model 1			Model 2		
	n	OR	95%CI (*P* value)	*n*	OR	95%CI (*P* value)
T2DM (diagnosis, fasting blood glucose > 126 mg/dL, or use of anti-diabetic drugs)	83	5.07	1.261-20.494 (0.022)	-		
IFG (fasting blood glucose > 100 mg/dL)	83	3.87	1.484-10.154 (0.006)	53	4.7[1]	1.280-17.316 (0.020)
Hypertriglyceridemia (triglycerides > 150 mg/dL)	96	0.27	0.069-0.967 (0.044)	-		
Low HDL levels	54	0.70	0.188-2.607 (0.595)	39	1.524[1]	0.185-12.588 (0.695)
Men: HDL ≤ 35 mg/dL						-
Women: HDL ≤ 39 mg/dL						
Obesity (BMI > 30 kg/m^2)	96	1.12	0.178-7.030 (0.91)	96	0.78[2]	0.115-5.339 (0.80)
Hypertension (defined by WHO)	95	1.176	0.379-3.626 (0.782)	62	1.92[1]	0.246-5.636 (0.458)
Hepatic steatosis determined by ultrasound	90	2.66	0.929-7.636 (0.068)	64	2.151[1]	0.555-8.33 (0.268)

[1]Adjusted for sex, age, and BMI; [2]Adjusted for sex, age, and genotype. The responders group is the reference group for all the dependent variables. Multivariable analysis could not be conducted owing to the small number of responders with *de novo* DM (*n* = 3) or small number of non-responders with hypertriglyceridemia (*n* = 3). OR: Odds ratio; BMI: Body mass index; IFG: Impaired fasting glucose; T2DM: Type 2 diabetes mellitus; HDL: High-density lipoprotein cholesterol; WHO: World Health Organization; DM: Diabetes mellitus.

crude ORs of 3.87 for IFG and 5.07 for T2DM (*P* = 0.006 and 0.022, respectively) in the unadjusted model, and a significant OR of 4.7 for IFG in the adjusted model (*P* = 0.02; model 2). Because of the low incidence of T2DM in responders (*n* = 3), T2DM could not be evaluated in the adjusted model (model 2; multivariable analysis) (Table 5). The crude OR for hypertriglyceridemia was 0.27 (*P* = 0.044). Because of the low occurrence of hypertriglyceridemia in the non-responders (*n* = 3), hypertriglyceridemia could not be evaluated in the adjusted model.

According to Kaplan-Meier analyses, there were lower rates of IFG and T2DM in responders than in the non-responders (*P* = 0.006 and 0.023, respectively) (Figure 1). Overall, the occurrence of MS in responders was not different from that in non-responders (Figure 2).

DISCUSSION

Our study, which aimed to examine the association between MS components and HCV infection based on treatment response and the influence of MS components on the success of antiviral therapy, did not detect any differences in most pre- or post-treatment MS component values between responders and non-responders to antiviral therapy. However, *de novo* IFG and T2DM occurred significantly more often in non-responders than in responders.

Our results regarding T2DM are consistent with those of other studies conducted with larger community cohorts. In a study conducted in Taiwan with a 7-year follow-up period, the cumulative incidence of T2DM was 14.3% in anti-HCV-positive patients and 8.6% in sero-negative individuals (*P* < 0.0001)[21]. In another study conducted in Japan, 143 of 2842 HCV-positive patients treated with IFNα monotherapy or IFNα and ribavirin combination therapy developed T2DM during a mean observation period of 6.4 years, and only 26 of these patients with T2DM had SVR[22].

HCV infection causes insulin resistance in the very early stage hepatic lesions (fibrosis stage 0 or 1). The progression of fibrosis, primarily owing to insulin resistance, worsens insulin resistance[23], which may lead to T2DM in predisposed individuals. In addition to SVR, fibrosis stage is independently associated with T2DM in HCV-infected patients[24]. Furthermore, a recent meta-analysis[25] and systematic review included 11 studies, of which only five examined the influence of

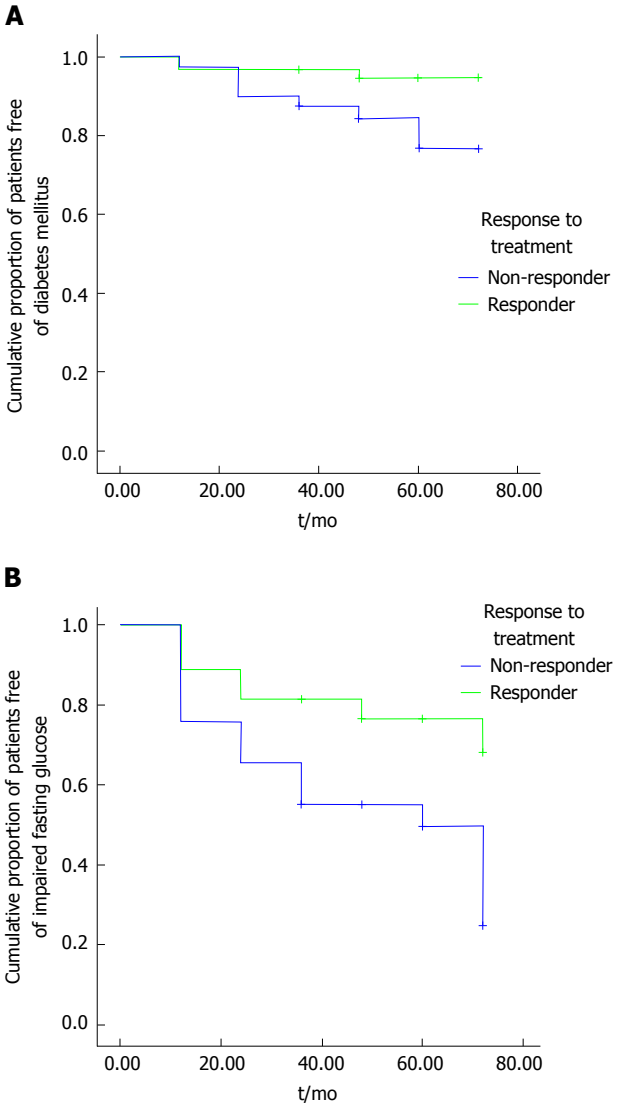

Figure 1 Kaplan-Meier analysis of the cumulative ratio of hepatitis C virus-positive patients. A: Without type 2 diabetes mellitus, based on the response to antiviral treatment; B: Without impaired fasting glucose, based on the response to antiviral treatment.

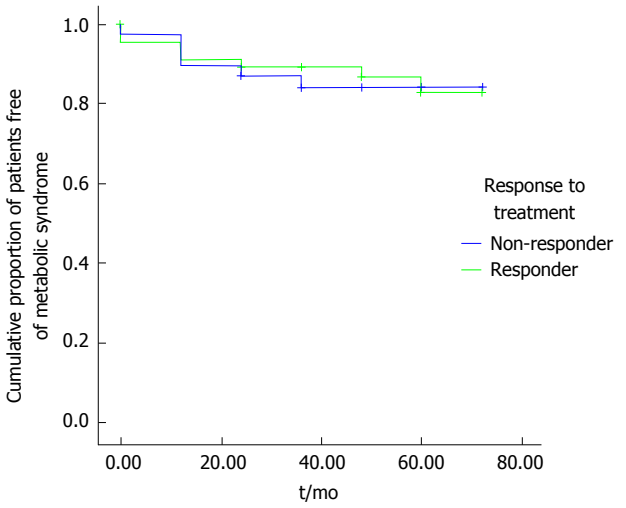

Figure 2 Kaplan-Meier analysis of the cumulative ratio of hepatitis C virus-positive patients without metabolic syndrome, based on the response to antiviral treatment.

HCV eradication on the risk of *de novo* glucose abnormalities[19,24,26,27] and insulin resistance[27]. Of the 2 studies with long follow-up periods [19,26], one (8-year follow-up) failed to demonstrate reduced *de novo* T2DM in patients with SVR[19]. The other (follow-up of 5.7 ± 2 years) showed that SVR reduced *de novo* glucose abnormalities in patients with chronic HCV. However, patient ages were not reported[26]. In another study with a relatively short follow-up (24 mo), SVR in patients with chronic HCV who did not have T2DM (51.8 ± 12.2 years old) prevented the development of *de novo* insulin resistance[28]. A significant two-third reduction in T2DM development was reported in a large cohort of patients with HCV and SVR (51.8 ± 9 years old) after IFNα monotherapy or combination therapy with IFNα and ribavirin[22]. Thus, curing HCV infections decreases the incidence of T2DM or improves homeostatic model assessment insulin resistance (HOMA-IR) in most studies[22-33]. These effects might be specific to patients with particular genotypes, with a reduction in HOMA-IR in patients with HCV genotype 1 but not those with genotypes 2/3[23]. Antiviral therapy might improve insulin resistance independent of virological outcomes[32] although a greater reduction in HOMA was observed in the patients who achieved persistent HCV clearance[33]. Antiviral therapy might also improve hepatic steatosis and fibrosis[29,34,35]. Thus, there might be an association between HCV and MS, and patients with HCV infection and MS have higher HOMA-IR values.

Several other components of MS, including waist circumference, BMI, and arterial hypertension, have been reported more frequently in non-responders to antiviral therapy[15]. The present study failed to demonstrate a significant relationship between baseline metabolic factors and treatment responses, potentially owing to the relatively young sample population. However, some metabolic factors (apart from T2DM) showed trends for differences based on the treatment response (*e.g., de novo* hypertriglyceridemia was 3.7 times more frequent in the responders than in the non-responders).

A bidirectional relationship between serum lipid levels and success of antiviral therapy for HCV has been reported[36]. Successful antiviral therapy might reverse the low LDL cholesterol, HDL cholesterol, and TG levels associated with HCV infection[7,12,13]. Low serum LDL levels in HCV infection result from the utilization of geranyl-geranyl phosphate, a product of the mevalonate pathway that is an early branch point of the cholesterol synthetic pathway, for HCV replication[1,7]. Higher baseline serum LDL cholesterol and lower serum TG levels were associated with higher rates of SVR[34], and lower serum LDL cholesterol levels correlated with low rates of SVR[33,35] in non-diabetic, non-cirrhotic patients infected with genotype 1 HCV. High serum LDL cholesterol levels

might improve the rates of SVR by competing with binding to hepatocyte LDL receptors and subsequently reducing the infection of hepatocytes with HCV[4,11,34]. In contrast, HDL cholesterol enhances HCV infection by facilitating its entry into hepatocytes[2]. However, high baseline serum HDL cholesterol levels reportedly interfere with the early viral response, but not with SVR[3,4], while serum HDL cholesterol inversely correlates with the rate of SVR in men but not in women, resulting in a lack of an association between overall baseline HDL levels and SVR[11].

Hepatic steatosis might also attenuate the antiviral treatment response, a trend that was demonstrated in the present study based on steatosis identified *via* liver biopsy. The insulin resistance and hepatic steatosis present during HCV infection are genotype-specific. Lower HOMA values are reported in patients infected with genotype 3 than in those infected with genotype 1. Insulin resistance-associated steatosis, which is present in patients with HCV genotype 3, is caused mainly by viral inhibition of the enzyme microsomal triglyceride transfer protein (viral steatosis), which might resolve with successful antiviral therapy. With the other HCV genotypes, steatosis is due to insulin resistance, stimulation of fatty acid synthesis, and inhibition of mitochondrial β-oxidation (metabolic steatosis)[37-39]. Metabolic steatosis might be associated with a high BMI and central obesity, which are not usually improved by viral eradication. HCV 1 and 4 core proteins might cause insulin resistance by functionally inhibiting insulin signaling pathways *via* increased levels of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and suppressors of cytokine signaling (SOCS) proteins, which impair insulin signaling and activate sterol regulatory element binding proteins, resulting in increased hepatic lipid synthesis[37-39]. The increased levels of hepatic proinflammatory cytokines have additional effect of negative regulate IFNα transduction.

This might explain the molecular link between insulin resistance and the nonresponse to antiviral therapy[4]. Furthermore, steatosis has been demonstrated to decrease SVR in HCV genotype 1 but not in HCV genotype 3, although steatosis is a predictor of HCV infection relapse with genotype 3 HCV[16]. This effect of steatosis on SVR might be the result of its association with insulin resistance[40], which is caused by excretion of TNFα and SOCS protein from the increased trunk fat in HCV infection. The lower level of PPAR-alpha mRNA also mediates genotype 3 hepatic steatogenesis[7,8,39]. However, Peg-INFα and ribavirin treatment response with HCV genotype 3 infection is better than with HCV genotype 1, despite more severe steatosis and lower cholesterol levels[10,37,40]. This might be related to the association between steatosis and higher BMI with genotypes 1 and 4[14,15].

It is noteworthy that, even in the era of direct acting antiviral (DAA; *e.g.,* telaprevir and boceprevir)-based triple therapy, some baseline metabolic variables might affect SVR, albeit to a lesser degree than with IFN and ribavirin combination therapy[41]. However, insulin resistance does not predict SVR to telaprevir-based triple therapy or to the protease inhibitor danoprevir monotherapy[16,42], while low serum LDL levels might affect SVR in telaprevir-treated patients, and obesity impairs SVR in patients treated with boceprevir-based regimen[16,41]. An additional link between DAA and MS has been demonstrated with danoprevir monotherapy, an inhibitor of NS3/3A HCV serine protease, which might increase insulin sensitivity considerably, independent of its antiviral effect[5,38,42]. Furthermore, DAAs are less effective against genotype 3 HCV infection, partly due to steatosis[43] and relapse after IFN-free therapy with the polymerase inhibitor sofosbuvir and ribavirin is associated with a low baseline LDL level.

This study has certain limitations. First, the study was retrospective, and some data were missing for some cases. Serum glucose levels were examined for diagnosis and monitoring glycemic control of T2DM and IFG. However, due to the retrospective nature of this manuscript, HbA1c and glycated albumin (GA) values were not available in most of the patients' charts. Nevertheless, liver cirrhosis and INF alpha treatment may falsely decrease HbA1c owing to hemolysis. On the other hand, GA as a glycemic control marker in patients with chronic liver disease may be overestimated, due to prolonged albumin half-life.

Additionally, the small sample size resulted in a small number of responders with *de novo* T2DM and non-responders with *de novo* hypertriglyceridemia, which limited the ability to assess these data in the multivariable logistic regression analysis.

However, the strengths of the study include the relatively young age of the patients and the relatively long follow-up period for all MS components after antiviral treatment, which enabled us to observe the effect of SVR on the cumulative incidences of IFG and T2DM. MS, including insulin resistance, hyperglycemia, high BMI, and liver steatosis, might complicate the disease course of patients infected with HCV, by enhancing cirrhosis, HCC, and cardiovascular disease[29,43-47]. Thus, it is worthwhile to screen these patients for T2DM[46,47]. Furthermore, with T2DM in the presence of HCV, there are specific considerations for antidiabetic treatment. Insulin and sulfonylurea administration might increase the risk of HCC[45,48], while the insulin sensitizers metformin and pioglitazone might decrease the risk of HCC and steatosis. However, these agents are harmful and might cause lactic acidosis and hepatic toxicity, respectively, in patients with liver cirrhosis[45]. The new dipeptidyl peptidase-4 inhibitors appear promising[45,47,49].

In conclusion, the results presented here suggest that MS components did not have any significant effect on the response to antiviral therapy, although hepatic steatosis tended to impair the response to antiviral treatment.

There were no differences in the post-treatment changes in most MS components between responders and non-responders to antiviral therapy. However, the incidences of *de novo* T2DM and IFG were significantly higher in non-responders. Given the younger age of the patient population in the present study compared to previous similar studies, the findings might suggest a direct effect of HCV on the development of T2DM independent of fibrosis or cirrhosis. The higher serum TG levels after SVR exemplify the interaction between HCV infection and lipid metabolic pathways. Due to the increased risk of HCV infection with T2DM, it might be appropriate to screen HCV patients for T2DM and insulin resistance and to consider treatment of T2DM in the presence of HCV with new antidiabetic agents.

COMMENTS

Background

A bidirectional association exists between chronic hepatitis C virus (HCV) infection and some components of metabolic syndrome (MS).

Research frontiers

Most, but not all, previous studies showed an association between chronic HCV infection and MS components, including type 2 diabetes mellitus (T2DM), insulin resistance, elevated body mass index, and hepatic steatosis. Several host MS components might affect the disease course and sustained virological response (SVR) of HCV-infected patients treated with pegylated-interferon α (Peg-IFNα) and ribavarin. On the other hand, SVR can affect some MS components, mainly decreased insulin resistance (IR) and decreased *de novo* occurrence of T2DM.

Innovations and breakthroughs

This study, which included a younger patient cohort, was designed to assess associations between MS components and HCV infection, but it did not show increased prevalence of T2DM with HCV infection. However, after long term follow-up T2DM was more frequent in non-responders to treatment with Peg-IFNα and ribavarin.

Applications

Patients with HCV infection should be frequently monitored for T2DM and treated appropriately, considering the increased risk of cirrhosis and HCC in young patients with HCV-associated diabetes mellitus. The authors think that further studies are needed to evaluate the mutual association of MS components with direct acting antiviral (DAA) drug therapy in chronic HCV infection.

Terminology

MS components refer to the metabolic syndrome factors; IR refers to insulin resistance; SVR refers to sustained virological response, which means viral eradication and cure; DAA refers to direct acting antiviral agents.

Peer-review

This is a very interesting study which shows the association between metabolic syndrome mechanism and response to antiviral treatment for chronic HCV infection. Patients were followed up for about 4 years; univariable and multivariable logistic regression analysis were applied. Data are well presented and discussed.

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ORIGINAL ARTICLE

Prospective Study

Reversibility of minimal hepatic encephalopathy following liver transplantation in Egyptian cirrhotic patients

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Abstract

AIM

To evaluate the reversibility of minimal hepatic encephalopathy (MHE) following liver transplantation (LT) in Egyptian cirrhotic patients.

METHODS

This prospective study included twenty patients with biopsy-proven liver cirrhosis listed for LT and twenty age- and sex-matched healthy control subjects. All underwent neuro-psychiatric examination, laboratory investigations, radiological studies and psychometric tests including trail making test A (TMT A), TMT B, digit symbol test and serial dotting test. The psychometric hepatic encephalopathy score (PHES) was calculated for patients to diagnose MHE. Psychometric tests were repeated six months following LT in the cirrhotic patient group.

RESULTS

Before LT, psychometric tests showed highly significant deficits in cirrhotic patients in comparison to controls (P < 0.001). There was a statistically significant improvement in test values in the patient group after LT; however, their values were still significantly worse than those of the controls (P < 0.001). The PHES detected MHE in 16 patients (80%) before LT with a median value of -7 ± 3.5. The median PHES value was significantly improved following LT, reaching -4.5 ± 5 (P < 0.001), and the number of patients with MHE decreased to 11 (55%). The pre-transplant model for end-stage liver disease (MELD) score ⩾ 15 was significantly related to the presence of post-transplant MHE (P = 0.005). More patients in whom reversal of MHE was observed had a pre-transplant MELD score < 15.

CONCLUSION

Reversal of MHE in cirrhotic patients could be achieved by LT, especially in those with a MELD score < 15.

Key words: Liver transplantation; Model for end-stage liver disease score; Psychometric tests; Minimal hepatic encephalopathy; Cirrhosis

Core tip: We evaluated the reversibility of minimal hepatic encephalopathy (MHE) following liver transplantation (LT) in Egyptian cirrhotic patients. Twenty patients with biopsy-proven liver cirrhosis listed for LT and twenty age- and sex-matched healthy controls were included. All underwent psychometric tests including trail making test A, trail making test B, digit symbol test and serial dotting test. Psychometric hepatic encephalopathy score was calculated for patients to diagnose MHE. Psychometric tests were repeated six months following LT in the cirrhotic patient group. We found that the reversal of MHE could be achieved by LT especially in those with a model for end-stage liver disease score < 15.

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INTRODUCTION

The prevalence of overt hepatic encephalopathy (HE) in patients with decompensated liver cirrhosis ranges from 16% to 21%, while that of minimal HE (MHE) or covert HE is 20%-80%[1].

MHE impairs daily functioning, driving performance, work capability and learning ability in cirrhotic patients. It also predisposes to overt HE and increased mortality[2]. There are several methods of diagnosing MHE, such as comprehensive neuropsychological examinations, standard psychometric batteries, and computerized testing[3].

The psychometric hepatic encephalopathy score (PHES) battery can detect neuropsychiatric abnormalities and MHE. It assesses visual perception, construction, visual/spatial orientation, motor speed and accuracy, concentration, and attention in cirrhotic patients with end-stage liver disease. When PHES was compared to the standard methods of determining HE, its sensitivity and specificity were 96% and 100%, respectively[4].

The PHES was initially composed of seven tests. The portosystemic encephalopathy battery was introduced later to exclude tests with poor sensitivity. It includes the line tracing test (LTT) and/or the digit symbol test, in addition to the number connection tests A and B (NCT A and B)[5]. The sum of the scores of these tests ranges between +5 and -15. A score of below or equal to -4 is diagnostic for MHE[4].

The LTT requires the longest time to calculate its score, and there is an existing controversy in interpretation of its two outcomes: Time and errors. Thus, only three of the four tests, NCT-A, NCT-B and DST, have been commonly used for MHE detection[6]. The result of any test was regarded to be abnormal if it was beyond the 2 standard deviation range of the control subjects. In some previous studies, MHE was diagnosed when two of these tests were abnormal[7]. In others, it was diagnosed when only one test was abnormal[8].

Liver transplantation (LT) is now considered an established effective and innovative treatment option for patients with end-stage liver diseases for a wide range of indications over the last fifty years[9]. The surgical outcomes and survival rates following LT have been previously estimated; however, the effect of LT on MHE has not been properly studied. A few studies have compared the cognitive performance of cirrhotic patients before and after LT. Some demonstrated cognitive improvement, and others have suggested reversibility of MHE after LT[10,11].

This study aimed to evaluate the reversibility of MHE following liver transplantation in Egyptian cirrhotic patients.

MATERIALS AND METHODS

This prospective study was conducted at Ain Shams Center for Organ Transplant, Ain Shams Specialized Hospital, Cairo, Egypt from June 2014 to April 2015. It included twenty right-handed patients with biopsy-proven liver cirrhosis listed for LT.

In addition, twenty age- and sex-matched healthy persons were enrolled, constituting the control group. The groups were similar regarding number of education years and handedness. The healthy controls were collected from the outpatient clinics among those coming for pre-employment screenings. Liver and systemic diseases were excluded by history, physical examination, laboratory and radiologic assessment.

Written informed consent was obtained from patients and controls prior to inclusion in the study. The study protocol was approved by the Research Ethical Committee of Faculty of Medicine, Ain Shams University according to the ethical guidelines of the 1975 Declaration of Helsinki.

Patients' selection

Exclusion criteria: (1) patients with clinical or laboratory evidence of any concomitant infection, severe gastrointestinal bleeding, anemia, electrolyte abnormalities, or renal insufficiency; (2) overt hepatic encephalopathy (persistent or episodic) as revealed by a standard clinical neurological examination; (3) significant cortical atrophy or other structural brain changes as revealed by conventional neurological imaging studies; (4) regular use of psychotropic drugs, such as benzodiazepines; (5) known major psychiatric disorder; (6) patients unable to perform the tests (illiterate or with upper limb motor handicaps); (7) less than 6 mo of complete alcohol abstinence; and (8) post-transplant toxic levels of immunosuppressive drugs.

All of the following were performed to recruited patients and controls: (1) full history taking together with a full clinical, neurological and psychiatric examination done by both an experienced hepatologist and neuropsychiatrist; (2) laboratory investigations including complete blood count; liver function tests: Alanine transaminase, aspartate transaminase, total and direct bilirubin, international normalized ratio (INR), prothrombin time, serum albumin; kidney function tests and full electrolytes including blood urea nitrogen, creatinine, sodium, potassium, magnesium, calcium, phosphorus; C-reactive protein to exclude patients with any infections; and post-transplant immunosuppressive drug levels for patients only to exclude those with toxic levels. The modified Child-Pugh score was calculated for patients, and each patient was categorized as A, B or C. Additionally, the model for end-stage liver disease (MELD) score was calculated for patients using laboratory results collected immediately before LT with no adjustments for malignancy. We calculated the MELD score using the following formula: MELD = [0.957 × ln(creatinine mg/dL) + 0.378 × ln(bilirubin mg/dL) + 1.12 × ln(INR) + 0.643 × 10^8]; (3) radiological studies included pelvi-abdominal ultrasound with examination of liver size, echogenicity, splenomegaly, amount of ascites, portal vein diameter and patency, presence of any hepatic focal lesions or any abdominal malignancy and a detailed kidney examination pre- and post-transplantation (Hitachi, EUB-5500). A computerized tomography (CT) for the brain was performed to all patients to exclude any brain pathology (Toshiba, High Speed 16 Slice); and (4) psychometric tests included the following neuropsychological tests.

Trail making test A: Patient should draw a line from number (1) to number (2) and from (2) to number (3) till reaching number (24), without elevating the pencil from the paper. The time was recorded in seconds. If the patient made an error, the examiner told him to correct it, but the timing was not stopped. The average score was 29 s, while the deficient score was > 78 s and the rule of thumb was that most completed it in 90 s. The rule of thumb is a broadly accurate guide or principle, based on practice rather than theory[12,13].

Trail making test B: Patient should draw a line from number (1) to letter (A), then from letter (A) to number (2), then from number (2) to letter (B), and so on, alternating the number and letter respecting the alphabetical order till letter (L). After explaining the test to the patient, timing should be started and recorded in seconds, including time needed to correct any error done. The average score was 75 s, while > 273 s was considered deficient and the rule of thumb was that most completed it in 3 min.

Digit symbol (substitution) test: A coding key was presented consisting of nine abstract symbols, each paired with a number. The patient was required to scan the key and write down the symbol corresponding to each number as rapidly as possible. Ninety seconds were given to the patient and when the time was finished, the number of symbols performed by the patient was counted. The score was recorded in points. If the patient made any errors, timing continued towards their 90 s, and the patient might lose time. A healthy individual should be able to complete the test in 90 s or less. A fall of 1 to 1.5 SD below the mean is considered suggestive of cerebral dysfunction.

Serial dotting test: Also called the circle dotting test, the serial dotting test (SDT) was used to test pure motor speed. The patient was asked to put a dot in each of the 100 circles given on the sheet after being prepared first by dotting the 20 circles at the top of the sheet.

The results of the trail making test A (TMT A), TMT B, and SDT were measured in seconds, including the time needed to correct any errors, and the results of digit symbol (substitution) test (DST) were measured as

points.

Accordingly, a better performance was reflected by a higher result of DST and lower results of other tests.

Interpretation of the score: To obtain the measure of overall visual-motor and visual-constructive performance, we calculated the average percentile score of the 4 selected tests: TMT A, TMT B, DST and SDT. The average score of these tests was arbitrarily named the visual-motor and visual-constructive performance (VMCP) score or PHES. The patient was diagnosed to have MHE when his total score was equal or below -4.

Post-transplantation follow-up

The immunosuppressive regimen included cyclosporine or tacrolimus, mycophenolate mofetil (MMF), and corticosteroids in all patients except those transplanted for hepatocellular carcinoma (HCC). In patients transplanted for HCC, the regimen included calcineurin inhibitors and steroids only. Trough levels of cyclosporine were maintained between 200 and 300 ng/mL while those of tacrolimus were maintained between 8 and 12 ng/mL. Rapid withdrawal of corticosteroids within three months was routine in all patients.

In cases of acute rejection, the first-line therapy consisted of optimization of the maintenance level of immunosuppression. If there was no response, then MMF or rapamycin were added to the patient's regimen, if not already being taken. In some cases, a shift from cyclosporine to tacrolimus was beneficial. A small dose of steroids was used if all other measures failed.

The complete psychometric battery was repeated six months following LT in the cirrhotic patient group. The post-transplant testing was done while the patient was in a stable condition with no clinical or laboratory evidence of any concomitant infection, anemia, electrolyte abnormalities, acute transplant rejection episode or other severe clinical problems.

Statistical analysis

Data were analyzed using the SPSS software computer program version 18 (SPSS, Chicago, IL, United States). They were described as the mean ± standard deviation (SD) for quantitative (parametric) variables and as median ± inter-quartile range (IQR) for quantitative (non-parametric) variables. Qualitative (categorical) variables were presented as frequency and percentage. The independent samples *t*-test was used for the comparison of quantitative parametric variables among two independent groups and the Mann Whitney *U* test was used for non-parametric data. The Wilcoxon Signed Ranks test was used for the comparison of quantitative non-parametric variables among two dependent groups (before and after transplantation). The χ^2 test (or Fisher's exact test when appropriate) was used for comparison of distribution of qualitative variables among different groups.

Significance level (*P*) value: (1) $P \leqslant 0.05$ was significant; (2) $P < 0.01$ was highly significant; and (3) $P > 0.05$ was non-significant.

The statistical methods of this study were reviewed by Azza M Hassan, Department of Community, Environmental and Occupational Medicine, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

RESULTS

This prospective study included twenty patients with biopsy-proven hepatitis C virus (HCV)-related liver cirrhosis listed for LT. Their mean age was 53.2 ± 5.39 years and they consisted of 17 males (85%) and 3 females (15%). Five patients were diagnosed with hepatocellular carcinoma on top of liver cirrhosis.

In addition, twenty age- and sex-matched healthy subjects were enrolled, constituting the control group. Their mean age was 53.4 ± 6.49 years and they consisted of 15 males (75%) and 5 females (25%).

Before LT, the median ± IQR of Child-Pugh score of the enrolled patients was 9 ± 4.5; five patients (25%) were Child A, six (30%) were B and nine (45%) patients were Child C. Their median ± IQR of MELD score was 14.5 ± 6.5, where 50% (10 patients) had a MELD score below 15% and 50% (10 patients) had a MELD score above 15.

Six months following LT, the median ± IQR of Child-Pugh and MELD scores were 6 ± 1.8 and 11.5 ± 4.5, respectively, with a statistically significant improvement ($P < 0.001$ and 0.002, respectively) (Table 1).

Table 2 shows the analysis of the median score values of different psychometric tests (TMT A, TMT B, DST and SDT) and the VMCP score in patients before and after LT, as well as in healthy control subjects. Before LT, the psychometric tests and the VMCP score showed highly significant deficits in cirrhotic patients in comparison to controls ($P < 0.001$).

After LT, there were statistically significant improvements in test values in the patient group when compared to their values before LT. However, the values of patients after LT were still significantly worse than those of the control subjects ($P < 0.001$).

Among the studied 20 cirrhotic patients, the PHES, represented by the VMCP score, detected MHE in 16 patients (80%) before LT, with a median value of -7 ± 3.5. The median PHES value was significantly improved following LT, reaching -4.5 ± 5 ($P < 0.001$), and the number of patients with MHE decreased to 11 (55%) post-LT.

Table 3 shows that the pre-transplant MELD score $\geqslant 15$ was significantly related to the presence of post-transplant MHE ($P = 0.005$). In cirrhotic patients with a pre-transplant MELD score $\geqslant 15$, 100% had pre-transplant MHE and 90% had post-transplant MHE. On the other hand, among those with a MELD score < 15, 60% had pre-transplant MHE and 20% had post-transplant MHE. A higher number of patients in whom reversal of MHE was observed had a pre-transplant MELD score < 15.

Table 1 Laboratory data, Child-Pugh and model for end-stage liver disease scores before and after liver transplantation in the patients' group

Variable	Before LT (median ± IQR)	After LT (median ± IQR)	*Z*	*P* value
INR	1.5 ± 0.6	1.4 ± 0.4	2.198	0.029[1]
ALT (N: 7-40 IU/L)	24.5 ± 35.25	19.5 ± 29.8	2.201	0.026[1]
AST (N: 7-37 IU/L)	44.5 ± 28	22.5 ± 13.75	2.918	0.002[2]
Total bilirubin (N: 0.2-1.2 mg/dL)	2 ± 2.45	1.4 ± 0.8	2.156	0.029[1]
Albumin (N: 3.5-5.3 g/dL)	2.3 ± 1.15	3.3 ± 0.9	3.021	0.021[1]
Creatinine (N: 0.5-1.2 mg/dL)	0.9 ± 0.4	1 ± 0.2	0.176	0.893
BUN (N: 20-40 mg/dL)	12 ± 6.8	11.5 ± 4.8	0.218	0.839
Sodium (N: 135-147 mEq/L)	132.5 ± 11.75	134.5 ± 10.8	0.197	0.856
Potassium (N: 3.5-5.3 mEq/L)	3.8 ± 0.7	4.4 ± 0.8	2.469	0.011[1]
Calcium (N: 9-11 mg/dL)	8.6 ± 1.1	8.9 ± 1.3	2.584	0.007[2]
Phosphorus (N: 3-4.5 mg/dL)	3.2 ± 0.6	3.6 ± 1.3	3.219	< 0.001[2]
Magnesium (N: 1.8-3.6 mg/dL)	2.1 ± 0.6	2.2 ± 0.7	1.777	0.076
Child-Pugh score	9 ± 4.5	6 ± 1.8	3.549	< 0.001[2]
MELD score	14.5 ± 6.5	11.5 ± 4.5	2.928	0.002[2]

[1]Significant; [2]Highly significant. *Z*: Wilcoxon signed ranks test; IQR: Inter-quartile range; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; MELD: Model for end-stage liver disease; LT: Liver transplantation; N: Normal range.

Table 2 Median score values of different psychometric tests in controls and patients before and after liver transplantation

	Controls	Patients before LT	Patients after LT	Patients before LT *vs* after LT (*P* value)[1]	Controls *vs* patients before LT (*P* value)[2]	Controls *vs* patients after LT (*P* value)[2]
TMT A (median ± IQR)	27 ± 8	110 ± 32.5	80 ± 30.8	0.010	< 0.001	< 0.001
TMT B (median ± IQR)	62 ± 17.3	282.5 ± 137.5	167.5 ± 72.0	0.002	< 0.001	< 0.001
DST (median ± IQR)	60 ± 4.75	22 ± 6	28.5 ± 14.5	0.001	< 0.001	< 0.001
SDT (median ± IQR)	34 ± 3.75	62 ± 20.75	51 ± 27.5	0.002	< 0.001	< 0.001
VMCP (median ± IQR)	1 ± 1	-7 ± 3.5	-4.5 ± 5.0	< 0.001	< 0.001	< 0.001

[1]Wilcoxon signed ranks test; [2]Mann Whitney *U* test. TMT: Trail making test; DST: Digit symbol test; SDT: Serial dotting test; VMCP: Visual-motor and visual-constructive performance score; LT: Liver transplantation; IQR: Inter-quartile range.

Table 3 Relation between pre-transplant model for end-stage liver disease score and the presence of pre- and post-transplant minimal hepatic encephalopathy *n* (%)

		Pre-transplant MELD score		χ^2	*P* value
		< 15 (*n* = 10)	⩾ 15 (*n* = 10)		
Pre-transplant	-ve	4 (40)	0 (0)	5.000[1]	0.087
MHE	+ve	6 (60)	10 (100)		
Post-transplant	-ve	8 (80)	1 (10)	9.899[1]	0.005[2]
MHE	+ve	2 (20)	9 (90)		

[1]Fisher's exact test; [2]Highly significant. MELD: Model for end-stage liver disease; MHE: Minimal hepatic encephalopathy; -ve: Negative; +ve: Positive.

Table 4 shows comparison between patients who recovered from MHE (*n* = 5) and those who didn't recover (*n* = 11) regarding age, sex, pre-transplant lab investigations and pre-transplant Child and MELD scores. We found that non-recovered patients had significantly higher INR, total bilirubin, Child and MELD scores than recovered ones (*P* = 0.027, 0.013, 0.038 and 0.009, respectively).

DISCUSSION

In the current study, twenty cirrhotic patients listed for LT and twenty healthy controls were included. Patients with pre or post-transplant clinical or laboratory evidence of infection, electrolyte imbalance, renal impairment or immunosuppressive drugs toxicity were excluded from the study. The etiology of liver cirrhosis in the included patients was chronic hepatitis C. Egypt has the highest prevalence of HCV worldwide, with an exceptionally high burden of liver disease[14].

A neuropsychological test battery, consisting of TMT A, TMT B, SDT and DST was applied to both the cirrhotic patient and control groups before and six months after LT. These are the same tests used by Wang *et al*[3] and Tsai *et al*[15] to diagnose MHE. They have high sensitivity and specificity and are easily applied with no difficulty in their score calculation[15,16]. These tests monitor changes in attention, motor speed and executive functions, which are the first to improve in the post transplantation period[3,16]. The TMT is a measure of attention, speed, and mental flexibility. It also tests spatial organization, visual pursuits, recall, and recognition[12]. Part A tests visual scanning, numeric sequencing, and visuo-motor speed, while part B tests cognitive demands including visual motor, visual spatial abilities and mental flexibility[16,17]. The DST measures the perceptual ability[18], while the SDT tests the pure motor speed[19].

The total score of the four tests (TMT A, TMT B, SDT

Table 4 Comparison between recovered and non-recovered patients regarding age, sex, pre-transplant laboratory investigations and pre-transplant Child and model for end-stage liver disease scores

Variable	Recovered (n = 5) (median ± IQR)	Non-recovered (n = 11) (median ± IQR)	Z[1]	P value
INR	1.5 ± 0.5	1.7 ± 0.5	2.231	0.027
ALT	25 ± 51	38 ± 36	0.397	0.743
AST	49 ± 45.5	53 ± 39	0.283	0.827
Total bilirubin	1.4 ± 1	3.1 ± 2.9	2.437	0.013
Albumin	2.3 ± 1	2.3 ± 0.5	1.208	0.267
Creatinine	0.7 ± 0.5	0.9 ± 0.4	0.517	0.661
BUN	11 ± 11.5	12 ± 5	0.514	0.636
Sodium	139 ± 12.5	132 ± 7	0.910	0.377
Potassium	4 ± 1.2	3.6 ± 0.8	1.310	0.221
Calcium	8.9 ± 0.7	8.3 ± 1.3	0.746	0.482
Phosphorus	2.7 ± 0.8	3.2 ± 1.3	1.204	0.259
Magnesium	2.3 ± 0.6	2 ± 0.6	0.969	0.355
Child-Pugh score	9 ± 1.5	11 ± 1	2.140	0.038
MELD score	13 ± 3.5	17 ± 5	2.557	0.009
Age (mean ± SD)	56.6 ± 1.7	51.2 ± 6.1	1.911[2]	0.077
Sex				
Male (n, %)	4 (80)	9 (81.8)	0.007[3]	1.000
Female (n, %)	1 (20)	2 (18.2)		

[1]Mann Whitney U test; [2]Independent samples t-test; [3]χ^2, Fisher's exact test. INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; MELD: Model for end-stage liver disease; IQR: Inter-quartile range; SD: Standard deviation.

and DST), which represents the VMCP score or PHES, has a cutoff level of -4. Any patient with a score below or equal to -4 is defined as having MHE[3,15,16].

With regard to the scores of TMT A, TMT B, DST and SDT, and the total PHES score in the current study; significant differences were found between patients and control subjects, together with the significant improvement in the patient scores after LT. This is in agreement with Mattarozzi *et al*[20].

The significant improvement of the patients' MELD score after LT agrees with Lin *et al*[10] and Mattarozzi *et al*[20].

The present study supports the relation between MHE and higher values of a MELD score. This is also in agreement with Mattarozzi *et al*[20] and Montagnese *et al*[21]. More patients with a pre-transplant MELD score > 15 experienced pre- and post-transplant MHE. More patients in whom reversal of MHE was observed had a pre-transplant MELD score < 15, indicating that early LT for patients with a MELD score < 15 may be associated with a higher incidence of reversal of MHE and could save the brain from the irreversible damages associated with end-stage liver disease. These findings may change the LT priority for patients with MHE with a MELD score < 15 receiving priority over those with a MELD > 15.

In a trial to find the factors affecting the reversibility of MHE, comparison between patients who recovered and those who didn't was done in the present study. Pre-transplant Child and MELD scores were significantly lower in patients who recovered from MHE. Age and sex differences were insignificant between those who recovered and those who didn't.

This is different from the study of Mechtcheriakov *et al*[22], in which the duration of liver cirrhosis and its severity (as determined by the Child classification) did not influence the improvement after LT. However, O'Carroll et *al*[23] reported that severe liver disease at pre-transplant assessment was associated with more slowing of reaction times and increased bioelectric dysfunction of the brain. In the study of Mechtcheriakov *et al*[22], patients' age was not related to recovery from MHE after LT which is similar to our study.

Although there was improvement in the cognitive function after LT in the current study, it did not reach the normal optimal levels of the healthy controls. This observation agrees with O'Carroll *et al*[23], Tarter *et al*[24] and Garcia-Martinez *et al*[25] indicating that MHE and the deterioration in cognitive function in liver disease patients are not completely reversible after LT.

It was hypothesized by Rose *et al*[26] that hepatic encephalopathy may be manifested by either "delirium-like" or "dementia-like" clinical features. The former is likely to be metabolic in origin, whereas the latter is likely to be due to a structural brain lesion, which may be specific to liver disease.

Ammonia has been suggested to have a role in the metabolic pathogenesis of MHE. Hyperammonemia in patients with liver cirrhosis may result in an increase in the brain glutamine with subsequent reduction in the brain magnetization-transfer ratio[25].

Teperman[11] demonstrated that patients who survived 10 years post-LT had significant cognitive dysfunction and poor health-related quality of life. This supports the evidence for a "dementia-like" parameter

of MHE that is irreversible after LT. Lin *et al*[10] showed improvement of both the extracellular cerebral edema and the demyelination of white matter in patients with MHE following LT, but they still did not reach the control level.

In the current study, gross structural brain lesions were excluded by CT brain before and after LT. Future studies should expand and should include larger sample size in order to investigate different metabolic, neurological and physical tests that could identify the exact causes of incomplete recovery of the brain cognitive functions.

In conclusion, the reversal of minimal hepatic encephalopathy in cirrhotic patients can be achieved by liver transplantation, especially in those with a pre-transplant MELD score < 15.

COMMENTS

Background

Minimal hepatic encephalopathy (MHE) impairs daily functioning, driving performance, work capability and learning ability in cirrhotic patients. It also predisposes to overt hepatic encephalopathy and increases mortality. The psychometric hepatic encephalopathy score (PHES) battery can detect neuropsychiatric abnormalities. It assesses visual perception, construction, visual/spatial orientation, motor speed and accuracy, concentration, and attention in cirrhotic patients with end-stage liver disease. Liver transplantation (LT) is now considered an established effective and innovative treatment option for patients with end-stage liver diseases. The effects of LT on MHE are poorly studied.

Research frontiers

The authors evaluated the reversibility of MHE following LT in Egyptian cirrhotic patients. Twenty right-handed patients with biopsy-proven liver cirrhosis listed for LT and twenty age- and sex-matched healthy control subjects were included. All underwent psychometric tests including trail making test A (TMT A), TMT B, the digit symbol test and the serial dotting test. The PHES was calculated to diagnose MHE. Psychometric tests were repeated six months following LT in cirrhotic patient group. They found that reversal of MHE in cirrhotic patients could be achieved by LT, especially in those with a MELD score < 15.

Innovations and breakthroughs

This is the first Egyptian study that addresses the reversibility of minimal hepatic encephalopathy following LT.

Applications

The findings of this study may represent a future strategy, indicating that early LT for patients with a MELD score < 15 may be associated with a higher incidence of reversal of MHE and could save the brain from the irreversible damage associated with end-stage liver disease.

Terminology

MHE is a neuropsychiatric syndrome that may occur in cirrhotic patients with no recognizable clinical symptoms of hepatic encephalopathy but with mild cognitive and psychomotor deficits, impairing daily functioning.

Peer-review

This study provides very interesting results which indicate that liver transplantation cannot fully recover all Egyptian patients from MHE caused by hepatitis C virus-induced cirrhosis, and has the most beneficial effect in patients with pre-transplant MELD score less than 15. This is the first study that investigated the reversibility of MHE in Egyptian population after liver transplantation, and provides important information regarding the effect of transplantation on the course of MHE.

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ORIGINAL ARTICLE

Prospective Study

Regulatory and activated effector T cells in chronic hepatitis C virus: Relation to autoimmunity

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Abstract

AIM

To investigate how Tregs are regulated in chronic hepatitis C virus (HCV) patients *via* assessment of Tregs markers (granzyme 2, CD69 and FoxP3), Teffs markers [TNFRSF4 (OX40), INFG] and *CD4*, *CD25* genes.

METHODS

A prospective study was conducted on 120 subjects divided into 4 groups: Group Ⅰ (n = 30) treatment naïve chronic HCV patients; Group Ⅱ (n = 30) chronic HCV treated with Peg/Riba; Group Ⅲ (n = 30) chronic HCV associated with non-organ specific autoantibody and Group Ⅳ (n = 30) healthy persons as a control group. Tregs and Teffs markers were assessed in peripheral blood mononuclear cells by quantitative real time reverse transcriptase-polymerase chain reaction.

RESULTS

Chronic HCV patients exhibited significant higher levels of both Teffs and Tregs in comparison to healthy control group. Tregs markers were significantly decreased in Peg/Riba treated HCV patients in comparison to treatment naïve HCV group. In HCV patients with antinuclear

antibody (ANA) +ve, Tregs markers were significantly decreased in comparison to all other studied groups. Teffs markers were significantly elevated in all HCV groups in comparison to control and in HCV group with ANA +ve in comparison to treatment naïve HCV group.

CONCLUSION

Elevated Tregs cells in chronic HCV patients dampen both $CD4^+$ and $CD8^+$ autologous T cell immune response. Interferon-α and ribavirin therapy suppress proliferation of Tregs. More significant suppression of Tregs was observed in HCV patients with autoantibodies favoring pathological autoimmune response.

Key words: Autoimmunity; T regulatory cells; Hepatitis C virus; T activator cells; Interferon

Core tip: A prospective study conducted on 120 subjects divided into: Treatment naïve hepatitis C virus (HCV) patients, HCV patients treated with old standard of care, HCV associated with antinuclear antibody (ANA) and healthy control group. Teffs/Tregs imbalance was evaluated. Results showed that HCV patients exhibited significant higher levels of both Teffs and Tregs markers. Interferon-α and ribavirin therapy suppresses proliferation of Tregs. More significant suppression of Tregs was observed in HCV patients with autoantibodies favoring pathological autoimmune response. Teffs markers were significantly elevated in HCV treated group and in HCV group with ANA +ve in comparison to treatment naïve HCV group.

Fouad H, El Raziky M, Hassan EM, Aziz GMA, Darweesh SK, Sayed AR. Regulatory and activated effector T cells in chronic hepatitis C virus: Relation to autoimmunity. *World J Hepatol* 2016; 8(30): 1287-1294 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i30/1287.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i30.1287

INTRODUCTION

Over 200 million people worldwide are suffering from chronic hepatitis C virus (HCV) infection and liver cirrhosis will be developed in about a quarter of these patients[1]. The prevalence rate of HCV genotype 4 in high risk populations in Egypt ranges from 73% to 90% and it was also found to be highly prevalent in sub-Saharan Africa and in the Middle East[2,3].

T lymphocytes play a major role in cell mediated immunity[4,5]. The several subsets of T cells have distinct functions and the majority is part of the adaptive immune system. Other subtypes can effectively present antigens to other T cells and are considered to be part of the innate immune system[6].

HCV is accompanied with different autoimmune manifestations[7], and could be a stimulator for the autoimmune reactions causing production of autoantibodies[8]. More recently, Acay *et al*[9] stated that the auto-antibodies in chronic HCV infection are highly incident. The authors stated that high percentages of patients with chronic hepatitis C had anti-mitochondrial antibodies, anti-smooth muscle antibodies, antinuclear antibody (ANA), thyroid antibody and anti-liver kidney microsomal antibodies.

The old HCV therapeutic protocol recommended by National Institutes of Health[10] was pegylated interferon (PEG-IFN) and ribavirin. Either endogenous or exogenous IFN-α leads to down regulation of $CD4^+$ $FoxP3^{hi}IFN\text{-}\gamma^{neg}$ activated T regulatory cells (aTregs) while at the same time induces induction of of $CD4^+$ $FoxP3^{low/neg}IFN\text{-}\gamma^{pos}$ T-activated cells (aTeffs). IFN-α play an essential role in suppression of Tregs *via* inhibition of interleukin-2 secretion[11].

Together, these observations support the fact that in early antiviral response there is a production of IFN-α which enhances CD4 effector functions by inhibiting Tregs activation, whereas sustained elevation of IFN-α reverses Tregs/Teffs balance towards Teffs activation, generation of auto antibody and development of autoimmunity.

The objective of the present study is to evaluate the extent of Teffs/Tregs imbalance in chronic HCV and its association with old standard of care as well as the presence of ANA.

MATERIALS AND METHODS

Study outcomes

Our research hypothesis was that HCV with or without IFN-α and ribavirin is usually associated with Tregs/Teffs imbalance with subsequent generation of autoantibodies.

The primary outcome for this study was to evaluate Teffs/Tregs balance and regulation in chronic HCV through assessment of Tregs markers (granzyme 2, CD69 and FoxP3), Teffs markers (TNFRSF4, INFγ) and *CD4*, *CD25* genes. Assessment of the effect of IFN-α and ribavirin on Teffs/Tregs balance as well as the association of Teffs/Tregs balance with the presence of antinuclear antibody were also conducted.

Study population

This was a prospective study conducted in Biochemistry and Molecular Biology Unit, Cairo University, Faculty of Medicine. The study included one hundred and twenty subjects categorized into 4 groups: Group Ⅰ (30 patients) treatment naïve chronic HCV patients; Group Ⅱ (30 patients) chronic HCV patients treated with the old standard of care therapy; Peg-IFN-α and ribavirin (Peg/Riba), group Ⅲ (30 patients) chronic HCV patients associated with non-organ specific autoantibody and group Ⅳ, 30 healthy persons served as a control group. The patients attended the Internal Medicine Department at Beni-Sueif General Hospital. Healthy controls matched the age and sex of other patients. Cairo University Institutional

Table 1 Primers for *TNFRSF4(OX40), granzyme 2, CD69, CD4, CD25, FoxP3* and *interferon γ* genes

TNFRSF4OX 40	Forward: '5 GCA ATA GCT CGG ACG CAA TCT 3'
DQ032625.1	Reverse: '5 GAG GGT CCC TGT GAG GTT CT 3'
Granzyme 2	Forward: '5 TAC CAT TGA GTT GTG CGT GGG 3'
NM_004131.4	Reverse: '5 GCC ATT GTT TCG TCC ATA GGA GA 3'
CD69	Forward: '5 GGT CAC CCA TGG AAG TGG TC 3'
NM_001781.2	Reverse: '5 GAC TTC GGA CCA CAG AGC AG 3'
CD4 NM_001195017.2	Forward: '5 CTG CAA GTT CTC ACA CCG TC 3'
	Reverse: '5 CTA GAG TTG CCT GCT CTG CC 3'
CD25 IL2R NM_000417.2	Forward: '5 GCT CTA CAC AGA GGT CCT GC 3'
	Reverse: '5 AGC ACA ACG GAT GTC TCC TG 3'
FoxP3	Forward: '5 CCC ATC CCC AGG AGT CTT G 3'
NG_007392.1	Reverse: '5 ACC ATG ACT AGG GGC ACT GTA 3'
Interferon γ	Forward: '5 ATG GTT GTC CTG CCT GCA AT 3'
NG_015840.1	Reverse: '5 CTT GCT TAG GTT GGC TGC CT 3'

review board in Faculty of Medicine approved the study. Informed written consent was signed by all subjects of the study.

The eligibility of selected patients included: (1) age between 18 and 65 years old; (2) anti-HCV positive serum; (3) positive HCV RNA detected by reverse-transcription/polymerase chain reaction (RT/PCR); (4) non-organ specific autoantibody by positive ANA test (titer > 1/32) in group Ⅲ only and < 1/16 in all other groups; and (5) white blood cell > 3.500/mm^3.

A signed informed consent was got in accordance with Declaration of Helsinki ethics guidelines.

Exclusion criteria include patients with: Hepatocellular carcinoma, HBV co-infection, severe psychiatric disease, HIV-positive patients, co-morbid serious conditions, schistosomiasis mansoni, past history of alcohol abuse or long use of hepatotoxic drugs.

All HCV-infected patients in the treated group had a 48 wk course of old standard of care (Peg/Riba therapy) and achieved sustained virologic response. The T cells markers were analyzed after more than 6 mo of the end of the Peg/Riba course.

Study analytic procedure

Whole blood was obtained from all subjects of the study. The mononuclear cell layer was isolated using Ficoll (Sigma, St. Louis, MO, United States) and centrifugation was conducted for 30 min at 400 g in cooling centrifuge.

RNA extraction: Total RNA was isolated from mononuclear cell layer using Qiagen purification reagent (Qiagen, CA, United States). The extracted RNA was quantified and checked for purity using a spectrophotometer (260/280 w.l.).

Primer sequence: PCR primers were got from GenBank RNA sequences cited at the following website: http://www.ncbi.nlm.nih.gov/tools/primer-blast (Table 1).

Real-time quantitative PCR using SYBR Green I: Step One plus real-time PCR system was used in the analysis using software version 3.1 (Applied Biosystems, United States). Optimization of the annealing temperature was conducted for the PCR protocol and for the primer sets.

All cDNA were prepared for all gene markers, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and for non-template negative control.

Five microliter of total RNA was used to generate cDNA using 20 pmol antisense primer and 0.8 μL AMV reverse transcriptase at 37 ℃ for 60 min. The relative abundance of mRNA species was evaluated using the SYBR® Green method (Applied Biosystems, CA, United States).

Annealing temperature of 60 ℃ was optimized for all primer sets. Real time polymerase reaction was performed in 25 μL reaction volume consisting of Mater Mix of SYBR Green, 3 μL of cDNA, 900 nmol/L of every primer. Amplification conditions were conducted according to the manufacturer specifications: 2 min at 50 ℃, 10 min at 95 ℃, 40 repeated cycles with 15 s denaturation and 10 min of annealing/extension at 60 ℃.

Calculation of relative quantification (relative expression)

The resulting data were expressed in Cycle threshold (Ct). The PCR data results show Ct values of all studied genes (*CD69, CD4, granzyme 2, TNFRSF4, FoxP3, CD25 and IFNγ*) and the house keeping gene (GAPDH). A negative control sample was no template cDNA was used. Target gene expression was related to GAPDH.

Data were calculated using the Applied Biosystems Step One plus software. Relative gene expressions of all assessed genes were calculated using the comparative Ct method. All values were normalized to GAPDH housekeeping gene and expressed as fold changes relative to the background levels found in the control samples.

Statistical analysis

Statistical Package of Social Studies (SPSS) version 16.0.1 (SPSS Inc., Chicago, IL, United States) was utilized. Numerical data were presented as mean ± standard deviation. The null hypothesis was calculated for multiple groups by a single-factor ANOVA and for two groups by

Table 2 Relative gene expression of Tregs and Teffs specific genes in the four groups

	Group Ⅰ (naïve)	Group Ⅱ (Peg/Riba)	Group Ⅲ (ANA+)	Group Ⅳ (control)
Granzyme 2 Tregs	0.704 ± 0.039	0.603 ± 0.046	0.400 ± 0.042	0.489 ± 0.053
CD69 Tregs	0.647 ± 0.037	0.54 ± 0.049	0.306 ± 0.036	0.383 ± 0.043
FoxP3 Tregs	0.531 ± 0.033	0.444 ± 0.046	0.330 ± 0.039	0.39 ± 0.030
TNFRSF4OX 40	0.596 ± 0.047	0.688 ± 0.057	0.707 ± 0.05	0.482 ± 0.056
CD4	0.584 ± 0.034	0.677 ± 0.048	0.712 ± 0.042	0.528 ± 0.05
CD25	0.595 ± 0.039	0.684 ± 0.053	0.73 ± 0.053	0.495 ± 0.041
Interferon γ	0.466 ± 0.035	0.483 ± 0.035	0.522 ± 0.044	0.357 ± 0.038

ANA: Antinuclear antibody.

Table 3 Multiple comparisons of gene expression of Tregs markers in the four studied groups

	Group Ⅰ naïve	Group Ⅱ Peg/Riba	Group Ⅲ ANA+	Group Ⅳ control
FOX3				
Group Ⅰ naïve		$^{a}P \leqslant 0.05$	$^{d}P \leqslant 0.001$	$^{b}P \leqslant 0.01$
Group Ⅱ Peg/Riba	$^{a}P \leqslant 0.05$		$^{a}P \leqslant 0.05$	$^{b}P \leqslant 0.01$
Group Ⅲ ANA+	$^{d}P \leqslant 0.001$	$^{a}P \leqslant 0.05$		$^{d}P \leqslant 0.001$
Group Ⅳ control	$^{b}P \leqslant 0.01$	$^{b}P \leqslant 0.01$	$^{d}P \leqslant 0.001$	
CD69				
Group Ⅰ naïve		$^{b}P \leqslant 0.01$	$^{b}P \leqslant 0.01$	$^{d}P \leqslant 0.001$
Group Ⅱ Peg/Riba	$^{b}P \leqslant 0.01$		$^{b}P \leqslant 0.01$	$^{d}P \leqslant 0.001$
Group Ⅲ ANA+	$^{b}P \leqslant 0.01$	$^{b}P \leqslant 0.01$		$^{d}P \leqslant 0.001$
Group Ⅳ control	$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$	
Granzyme				
Group Ⅰ naïve		$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$
Group Ⅱ Peg/Riba	$^{d}P \leqslant 0.001$		$^{b}P \leqslant 0.01$	$^{d}P \leqslant 0.001$
Group Ⅲ ANA+	$^{d}P \leqslant 0.001$	$^{b}P \leqslant 0.01$		$^{d}P \leqslant 0.001$
Group Ⅳ control	$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$	$^{d}P \leqslant 0.001$	

ANA: Antinuclear antibody.

unpaired *t*-test. Statistically significant was considered if *P* value was < 0.05.

RESULTS

This study included 120 subjects divided into four groups. There weren't any difference between the four groups with statistical significance regarding age, sex distribution, albumin and T.bilirubin values (*P*-value > 0.05).

Findings of the present study exhibited that chronic HCV patients exhibited significant higher levels of both Teffs and Tregs markers as compared to healthy control group. Tregs markers (granzyme 2, CD69, FoxP3) were significantly decreased in Peg/Riba treated HCV patients in comparison to treatment naïve HCV group (Tables 2 and 3).

In HCV patients with autoantibodies, Tregs markers were significantly decreased in comparison to all the other studied groups (Tables 2 and 3, Figure 1).

Teffs specific genes (*TNFRSF4* and *IFN-γ*) and *CD4, CD25* showed significant elevation in treatment naïve HCV group in comparison to control group (Tables 2 and 4, Figure 2).

More significant elevation in *Teffs* genes was observed in both Peg/Riba treated HCV and HCV with autoantibodies groups as compared to treatment naïve HCV and control groups (Tables 2 and 4, Figure 2).

DISCUSSION

HCV is reported to suppress immune system to sustain chronic infection. Accumulation of Tregs and activation of inhibitory signaling pathways play essential roles in suppressing antiviral effector T cells (Teffs). The mechanisms by which HCV impairs Teffs include: Induction of Tregs, Th1 deficiency or Th2 dominance, blunted T cell activation, T cell apoptosis and T cell anergy[12].

The objectives of the present study were to assess the extent of upregulation of Tregs in HCV patients whether or not associated with auto-antibodies. In the present study, we have evaluated certain markers of Tregs and Teffs in peripheral blood mononuclear cells.

FOXP3 (forkhead box P3) is a member of forkhead/winged-helix family of transcriptional regulators which are master regulators in the development and function of Tregs[13,14].

Our results exhibited significant upregulation of FOXP3 in all HCV patients groups as compared to healthy controls. Other studies also confirmed accumulation of $FOXP3^{+}$ Tregs in most chronic viral infections with subsequent suppression of antiviral $CD4^{+}$ and $CD8^{+}$ T cell responses[15-18].

Moreover, findings of our study demonstrated more significant decrease in Tregs specific genes (*CD69*, *FoxP3*, and *granzyme 2*) in HCV patients group after Peg/

Table 4 Multiple comparisons of gene expression of Teffs markers in the four studied groups

	Group Ⅰ naïve	Group Ⅱ Peg/Riba	Group Ⅲ ANA+	Group Ⅳ control
TNFRSF4OX 40				
Group Ⅰ naïve		[b]$P \leqslant 0.01$	[a]$P \leqslant 0.05$	[a]$P \leqslant 0.05$
Group Ⅱ Peg/Riba	[b]$P \leqslant 0.01$		NS ($P > 0.05$)	[b]$P \leqslant 0.01$
Group Ⅲ ANA+	[a]$P \leqslant 0.05$	NS ($P > 0.05$)		[d]$P \leqslant 0.001$
Group Ⅳ control	[a]$P \leqslant 0.05$	[b]$P \leqslant 0.01$	[d]$P \leqslant 0.001$	
CD4				
Group Ⅰ naïve		[b]$P \leqslant 0.01$	[a]$P \leqslant 0.05$	[a]$P \leqslant 0.05$
Group Ⅱ Peg/Riba	[b]$P \leqslant 0.01$		NS ($P > 0.05$)	[a]$P \leqslant 0.05$
Group Ⅲ ANA+	[a]$P \leqslant 0.05$	NS ($P > 0.05$)		[b]$P \leqslant 0.01$
Group Ⅳ control	[a]$P \leqslant 0.05$	[a]$P \leqslant 0.05$	[b]$P \leqslant 0.01$	
CD25				
Group Ⅰ naïve		[b]$P \leqslant 0.01$	[a]$P \leqslant 0.05$	[a]$P \leqslant 0.05$
Group Ⅱ Peg/Riba	[b]$P \leqslant 0.01$		NS ($P > 0.05$)	[b]$P \leqslant 0.01$
Group Ⅲ ANA+	[a]$P \leqslant 0.05$	NS ($P > 0.05$)		[d]$P \leqslant 0.001$
Group Ⅳ control	[a]$P \leqslant 0.05$	[b]$P \leqslant 0.01$	[d]$P \leqslant 0.001$	
Interferon-γ				
Group Ⅰ naïve		[b]$P \leqslant 0.001$	[b]$P \leqslant 0.001$	[a]$P \leqslant 0.05$
Group Ⅱ Peg/Riba	[b]$P \leqslant 0.001$		NS ($P > 0.05$)	[a]$P \leqslant 0.05$
Group Ⅲ ANA+	[b]$P \leqslant 0.001$	NS ($P > 0.05$)		[b]$P \leqslant 0.01$
Group Ⅳ control	[a]$P \leqslant 0.05$	[a]$P \leqslant 0.05$	[b]$P \leqslant 0.01$	

ANA: Antinuclear antibody; NS: Non significant.

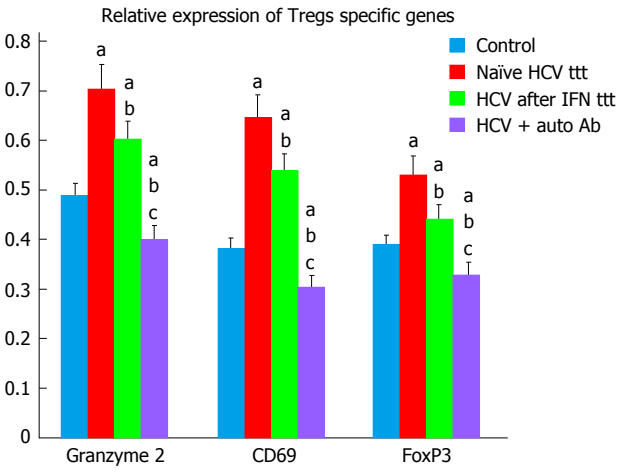

Figure 1 Relative gene expression of Tregs specific genes in the studied groups. [a]Significant difference *vs* control group; [b]Significant difference *vs* treatment naïve HCV group; [c]Significant difference *vs* HCV group after treatment. HCV: Hepatitis C virus; IFN: Interferon.

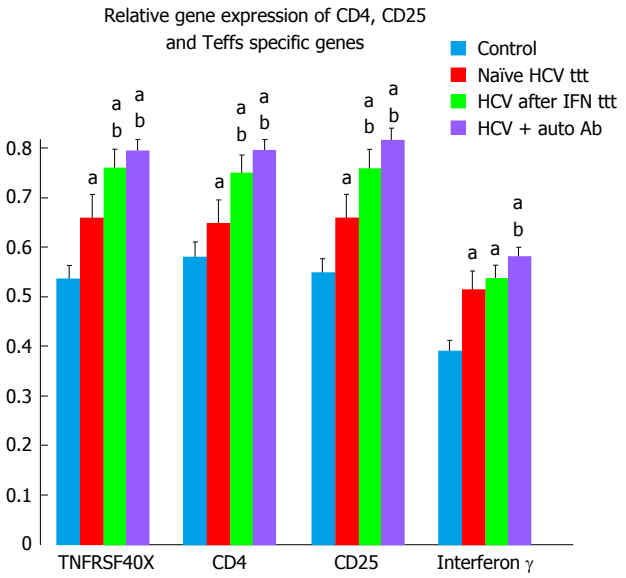

Figure 2 Relative gene expression of Teffs specific genes and CD4, CD25 in the studied groups. [a]Significant difference *vs* control group; [b]Significant difference *vs* treatment naïve HCV group. HCV: Hepatitis C virus.

Riba therapy. Similar findings were reported by Langhans *et al*[19] who stated that ribavirin can inhibit functions of HCV-specific Tregs beside its immuno-stimulatory effects on TH1 cells. Ribavirin can subsequently inhibit Treg-mediated suppression of Teffs in chronic HCV infections pushing the disease towards autoimmune responses.

Golding *et al*[11] stated that IFN-α, promotes proliferation of FoxP3$^{Low/Neg}$IFN-γ^{Pos} activated Teffs while simultaneously suppresses the development of FoxP3^{HI}IFN-γ^{Neg} activated Tregs. These data coincided with our findings in group Ⅱ in relation to the other groups.

CD4 gene product is a membrane glycoprotein of T lymphocytes that mediates initiation and augments early phase of T-cell activation[20-22]. Findings of the present study demonstrated significant elevation of *CD4* gene in all HCV groups in comparison to control group suggesting increase in activated T cells function. More significant elevation of CD4 was observed in HCV after treatment and in HCV with auto-antibodies groups.

CD25 is a type Ⅰ transmembrane protein present on activated T cells, activated B cells and in memory CD8 T cells[23-27]. Our results demonstrated significant elevation of CD25 in all HCV studied groups with more significant elevation after Peg/Riba therapy. Similar findings were reported by Caetano *et al*[28] in chronic HCV patients during Peg/Riba treatment who presented an amplified CD8 T-cell responses specific to HCV and more increase Teffs.

Moorman *et al*[12] showed that many inhibitory signaling pathways were up-regulated during chronic HCV infection, resulting in expansion of Tregs and contraction of Teffs. Thus, this inhibitory pathway may not only regulate proliferation and differentiation of naïve T cells, but also control responses of Teffs, memory cells, and expansion of Tregs[29].

These facts coincided with our results that showed significant elevation of Tregs specific genes (*CD69*, *FoxP3*, and *granzyme 2*) and significant elevation of Teffs specific genes (*TNFRSF4*, *INF-γ*) and *CD4*, *CD25* genes in both groups of HCV whether naïve or after treatment in comparison to healthy controls.

Granzyme B encodes a protein that is essential in induction of cell-mediated immune response for the faster initiation of target cell apoptosis by cytolytic T lymphocytes[30]. Tregs possess granzyme B, enabling them to induce apoptosis in effector T-cells[31,32]. Our results demonstrated that granzyme B was found to be significantly elevated in naïve HCV patients with significant decrease after Peg/Riba therapy. More significant decrease in its levels was observed in HCV patients with autoantibodies favoring autoimmune response in those patients.

CD69 expression was studied by Colbeck *et al*[33] and they stated that Tregs expressing CD69(+) are more proliferative and more suppressive than their CD69(-) counterparts. This finding explains our results that showed significant lower CD69 expression in HCV patients with autoantibodies suggesting inhibition of Tregs activity favoring autoimmune environment.

IFN-γ is a member of the type Ⅱ class of interferons[34]. Longhi *et al*[31] stated that $CD8^+$ T cells when cultured on their own secrete much higher levels of IFN-γ in patients with autoimmune hepatitis when compared to normal subjects and have a high proliferation rate. This finding coincided with our results that demonstrated significant higher levels of IFN-γ in patients with autoantibodies than healthy controls.

The protein encoded by the *TNFRSF4* gene belongs to tumor necrosis factor - receptor superfamily that has essential roles in $CD4^+$ T cell response[35,36].

Our findings showed that, in HCV patients with ANA +ve, *TNFRSF4* and *IFN-γ* (T effector gene) were significantly higher as compared to HCV naïve and healthy control groups. This means that T effector cells dominate over T regulatory cells favoring autoimmunity and initiating pathological immune response with production of autoantibodies. Similar findings were reported by González-Amaro *et al*[37].

Also, our results agreed with similar findings reported by Longhi *et al*[31] who stated that regulatory T cells are decreased numerically and impaired functionally in autoimmune hepatitis. Similar to our results, several studies reported that HCV infection induces a dramatic increase in Tregs, which contributes to the immune response failure during HCV infection[38].

In conclusion, chronic HCV patients exhibited significant higher levels of both Teffs and Tregs in comparison to healthy controls. Moreover, elevated levels of Treg cells in patients with chronic HCV dampen both the $CD4^+$ and $CD8^+$ autologous T cell immune response. IFN-α and ribavirin therapy suppress proliferation of Tregs and do not restore the Teffs/Tregs imbalance. More significant suppression of Tregs was observed in HCV patients with autoantibodies favoring pathological autoimmune response.

COMMENTS

Background

Hepatitis C virus (HCV) is accompanied with different autoimmune manifestations, and could be a stimulator for the autoimmune reactions causing production of autoantibodies. More recently, Acay *et al* stated that the auto-antibodies in chronic HCV infection are highly incident. The authors stated that high percentages of patients with chronic hepatitis C had anti-mitochondrial antibodies (AMA), anti-smooth muscle antibodies (ASMA), anti-nuclear antibodies (ANA), thyroid antibody and anti-liver kidney microsomal antibodies (anti-LKM-1). The old HCV therapeutic protocol was pegylated interferon (IFN) and ribavirin. IFN-α leads to down regulation of $CD4^+$ $FoxP3^{hi}IFN\text{-}\gamma^{neg}$ activated T regulatory cells (aTregs) while at the same time induces induction of of $CD4^+$ $FoxP3^{low/neg}IFN\text{-}\gamma^{pos}$ T-activated cells (aTeffs). Together, these observations support the fact that sustained elevation of IFN-α reverses Tregs/Teffs balance towards Teffs activation, generation of auto antibody and development of autoimmunity. The objective of the present study is to evaluate the extent of Teffs/Tregs imbalance in chronic HCV and its association with old standard of care as well as the presence of ANA.

Research frontiers

IFN-α/ribavirin old therapeutic protocol enhances CD4 effector (Teffs) functions by inhibiting Tregs activation. The old protocol reverses Tregs/Teffs balance towards Teffs activation, generation of auto antibody and development of autoimmunity.

Innovations and breakthroughs

New direct acting antiviral drugs do not induce Tregs/Teffs imbalance, whereas the old standard of care IFN-α and ribavirin induce Tregs/Teffs imbalance. Replacing the old therapeutic protocol by the new direct acting antiviral drugs is mandatory because beside its efficacy, the new direct acting antiviral drugs do not induce autoimmunity.

Applications

Chronic HCV patients exhibited significant higher levels of both Teffs and Tregs in comparison to healthy controls. Moreover, elevated levels of Treg cells in patients with chronic HCV dampen both the $CD4^+$ and $CD8^+$ autologous T cell immune response. IFN-α and ribavirin therapy suppress proliferation of Tregs and do not restore the Teffs/Tregs imbalance. More significant suppression of Tregs was observed in HCV patients with autoantibodies favoring pathological autoimmune response. Repalcing the old therapeutic protocol; IFN-α and ribavirin by the new direct acting antiviral drugs is mandatory and is also essential avoid Teffs/Tregs imbalance.

Terminology

Tregs also known as suppressor T cells, are a subpopulation of T cells that down-regulates or suppress induction, proliferation and activation of effector T cells. Tregs also maintain tolerance to self-antigens and prevent autoimmunity. Teffs includes various T cell types that actively respond to antigenic stimuli, such as co-stimulation. This includes helper T cells, cytotoxic or killer T cells, and potentially other T cell types as memory cells. HCV-induced autoantibodies involves: AMA, ASMA, ANA, thyroid antibody and anti-LKM-1. Forkhead box P3 is a member of forkhead/winged-helix transcriptional regulators which master the development and function of Tregs. *CD4* gene product is a membrane glycoprotein of T lymphocytes that mediates initiation and augments early phase of T-cell activation. Granzyme B is a protein that enables Tregs to induce apoptosis in effector T-cells. *TNFRSF4* gene belongs to tumor necrosis factor

-receptor superfamily that plays essential roles in CD4$^+$ T cell response.

Peer-review

The manuscript presents the gene expression of immune molecules in peripheral blood mononuclear cell by reverse transcription polymerase chain reaction. And to investigate the immune changes induced by HCV, as well as after PR therapy. This paper may be interest to the readers of the journal and provide some knowledge for clinicians and researchers.

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