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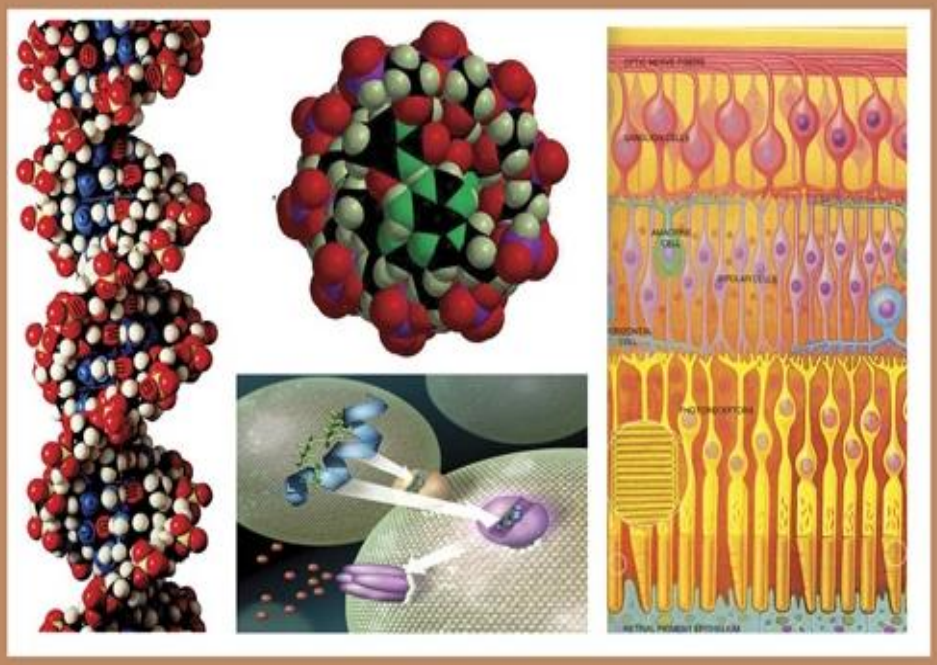

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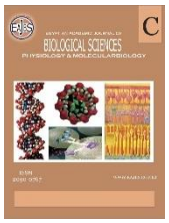

Gene Expression Profiling Unravels Hepatitis C Virus (HCV) Infection-induced Temporal Alteration of Gene Expression in Hepatocellular Carcinoma (HCC)

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ABSTRACT

Hepatitis C virus-induced liver cirrhosis and hepatocellular Carcinoma (HCC) is a global health concern. Underlying molecular mechanism of HCV-induced-HCC by temporal expression mRNA profiling of mock and HCV-infected Huh7.5.1dif cells was unraveled. A catalogue of bio-markers of HCC was analyzed to interpret the clinical relevance of the bio-markers. The percentage of over-expression and co-occurrence for targeted bio-markers within The Cancer Genomic Atlas (TCGA) were mapped using cBioPortal for cancer genomics. Spotting of differentially expressed genes (DEGs) and related temporal pathways enrichment analysis was accomplished employing MATLAB functions (e.g. mattest). Module analysis of the designed co-expression network using Cytoscape software was carried out. The overall expression of targeted bio-markers was recorded in 60% of the cases. Significantly overexpressed biomarkers recognized were CLK2, E2F5, CDK5, E2F3, MCM3, PCNA and CDK4. CCNB2, CLK2, CDK4, CDC7, E2F3, PCNA, and MCM3 predominantly controlled the co-expression of the listed bio-markers. After the initial phase of infection with a detrimental expression changing pattern, stability in expression followed by consistency in the level of expression of a set of genes was observed. Over-expressed screened biomarkers encompass the potential to be the candidate molecular target for surveillance, diagnosis and therapy of fetal HCV- induced-HCC.

INTRODUCTION

Liver cancer (hepatic cancer) is considered to be frequently diagnosed fetal disease and it is one of the major etiology of global cancer-associated mortality and morbidity (Center and Jemal 2011; Jemal *et al.,* 2011)**.** The most frequent histological subtype of liver cancer is lethal hepatocellular carcinoma (HCC) which stems from hepatocytes and accounts for more or less seventy to eighty-five percent of all cases of hepatic cancer (Perz *et al.,* 2006)**.** Hepato- carcinogenesis and its progression is a lengthy multi-factorial process that contributes to the heterogeneity of hepatocellular carcinoma (HCC) (Thorgeirsson and Grisham 2002)**.** Hepatitis C Virus (HCV) and Hepatitis B virus (HBV) in their infection in a chronic stage is the principal etiology (in 80% of cases) of the pathogenesis and progression of HCC (Beasley *et al.,* 1981; Bruix *et al.,* 2004; Cougot *et al.,* 2005)**.** But there are some other low-incidence causal elements for HCC too which include alcoholic abuse, non-alcoholic fatty hepatic (liver) diseases, exposure to aflatoxin B1, iron over-burden, obesity, smoking, diabetes, hereditary hemochromatosis, alpha1-antitrypsin-deficiency disease, autoimmune hepatitis, a few porphyrias (inherited disorders of blood), and a genetic Wilson's disease (El-Serag 2011; Llovet *et al.,* 2003)**.**

The unavailability of the HCV vaccine and limited options for therapeutic management of HCV infection and its progression to liver cirrhosis and HCC raise a growing concern. HCV-triggered progression of HCC viral genotype-dependent and is influenced by the disease period spanning over approximately twenty to forty years (Bruno *et al.,* 2007; Vescovo *et al.,* 2016)**.** The progression of HCV-associated carcinogenesis is mediated by a combination of host-triggered immune response and viral-induced factors. Lipogenesis and impairment of oxidative stress metabolism may be driven by HCV element (viral core protein), though the data of evidence of the oncogenic effect (direct) of HCV of hepatocytes is restricted to animal models (Li *et al.,* 2007)**.** HCV proteins (core and structural proteins) constitute a vital impact of the infection by causing chronic inflammation, immunological response-mediated disorders and death of hepatocytes, fibrosis and multilevel disorders (cellular pathways, such as proliferation, apoptosis and DNA repair) that head to the development of HCC. HCV exhibits sophisticated strategies for temporal alteration of key metabolic pathways and regulatory circuits of the cell (cell signaling pathways, RNA interferences and translational machinery) together with evasion of antiviral responses (Lupberger *et al.,* 2015; Lupberger *et al.,* 2011; Mailly *et al.,* 2015; Van Renne *et al.,* 2018)**.** Up-regulation of cell proliferation and growth following activation of key signaling pathways or inhibition of cellular checkpoints and tumor suppressor genes (specifically retinoblastoma protein and p53 tumor suppressor) is induced by the direct action of Hepatitis C Virus (HCV) viral proteins for development, promotion and progression of hepatocellular carcinoma (Lemon and McGivern 2012; Okuda *et al.,* 2002)**.** Synergistic loss of tumor suppressor (p53) and retinoblastoma eventually leads to a greater extent of carcinogenesis (Okuda *et al.,* 2002)**.** Considerable accumulation of genetic alteration in patients with cirrhosis induces the repeated cell cycles that head to the hepatocytes transformation to the malignancy (HCC tumorigenesis). The significant genetic aberrations in the case of HCCs encompasses mutated tumor protein p53 (TP53), β-catenin (CTNNB1) and the gene locus encoding telomerase reverse transcriptase (Tomasetti *et al.,* 2017)**.** These substantial mutations pose threats to maintenance of telomere which heads to enhanced oxidative stress. Substantial accumulation of mutations in hepatocytes (in cirrhotic nodules) rendering the cell's re-entry to the cell cycle, reactivation of telomerase and their progression by way of cancer (tumor) check-points in majority of the cases of HCC (Nault *et al.,* 2014)**.** Moreover, HCV as well as host immune response-induced oxidative stress on liver cells (hepatocytes) gives rise to death of the cells, regeneration, accumulation of genetic alteration in the hepatocytes, and eventually development and progression of HCC. Various existing scientific literature reflects that HCV infection is a potential causal factor of liver ailments for instance steatosis, fibrosis, cirrhosis and hepatocellular carcinoma. A comprehensive understanding of the genetic basis of the pathogenesis of HCV infection, liver disease progression and landscape of carcinogenesis is lacking. Moreover, the significant drivers in hepatocellular carcinoma (HCC) tumorigenesis are not considerably apprehended and thus, necessitating further investigations into it. Lately, the genetic landscape of human diseases has been unraveled owing to a paradigm shift in the field of bio-medical research created by next generation sequencing (NGS) approaches (Meyerson *et al.,* 2010; Shendure and Ji 2008)**.** In the recent decades, application of NGS for further delineation of genetic basis of cancer and determination of genetic mutations (novel) that are key drivers of tumorigenesis (development) and progression of cancer, especially HCC have been emphasized to a greater length (Schulze

et al., 2015; Totoki *et al.,* 2014). The diversity of transcriptional alterations arising in the carcinogenesis of the liver at a greater level has been delineated by various investigations on gene expressions in HCC (Hoshida *et al.,* 2010). Commencing from a collection of studied known bio-markers of HCC and a publicly available dataset for hepatocellular carcinoma (temporal data) with and without HCV infection. Biomarkers and their clinical relevance in HCC, analysis of significantly over-expressed HCC biomarkers, differentially expressed genes (DEGs) along with the analysis of enriched pathways from the gene expression data were aimed to be investigated in the present study.

MATERIALS AND METHODS

In the current investigation, genes of clinical relevance as HCC bio-markers, over-expression of HCC biomarkers, and differentially expressed genes together with enriched pathways retrieved from analysis of data of expressed genes which was acquired from Gene Expression Omnibus were thoroughly studied by applying computational approaches and integrating experimental data to unravel the critical genes and the pathways likely associated with human HCC. The different analytical approaches applied have been outlined in a workflow diagram (Fig. 1).

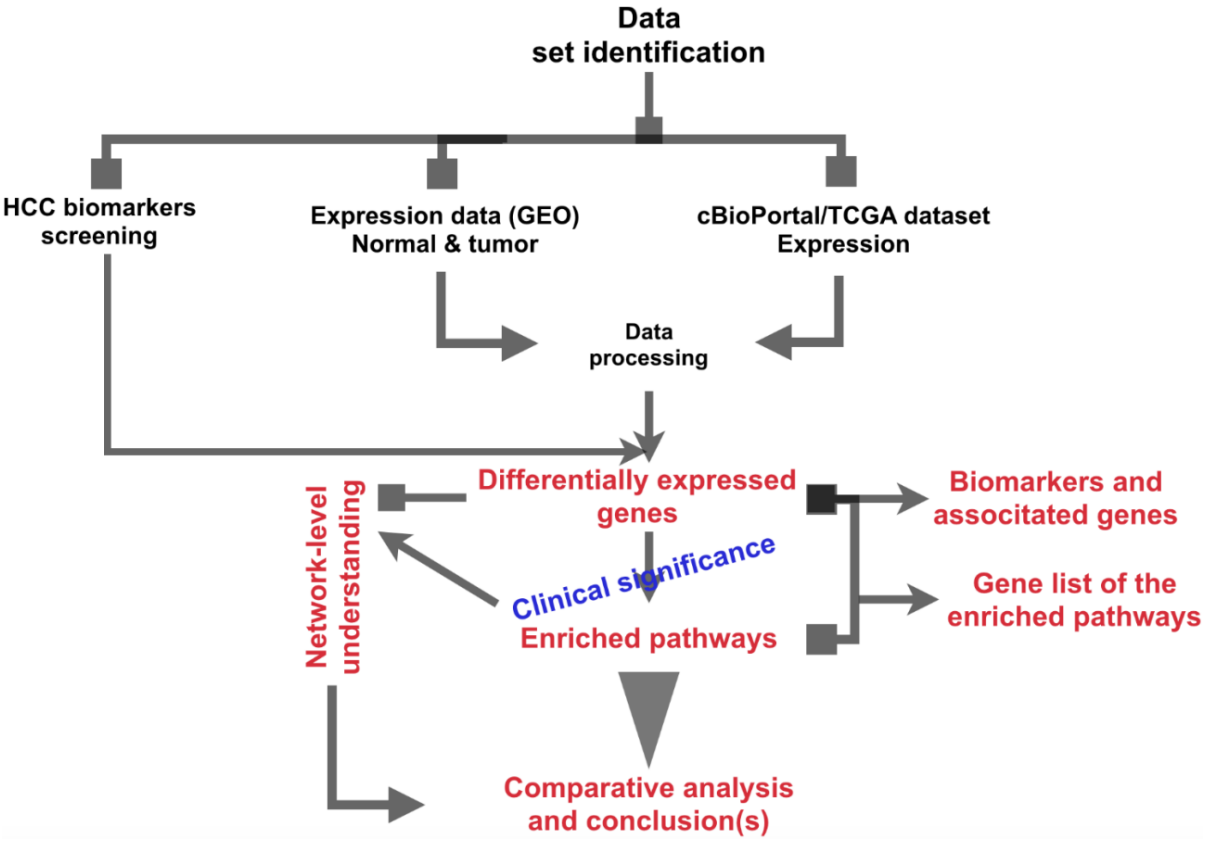

Fig. 1. Methodological workflow of the current study.

Acquisition of HCC Bio-Markers (gene signature) and Study Of Their Clinical Relevance:

HCC bio-markers were selected and processed (Andrisani *et al.,* 2011). Briefly, the percentage of over-expression (both individually and overall) and co-occurrence for the selected genes (bio-markers) within The Cancer Genomic Atlas (TCGA) database were mapped out by using cBioPortal for cancer genomics in HCC. After analyzing the clinical significance of the over-expressed HCC bio-marker genes in the samples, the co-expression network was drawn using Cytoscape tools (Su *et al.,* 2014).

Temporal Gene Expression Profiling And Functional Relevance:

Genome-wide mRNA expression data (mock and HCV-infected Huh7.5.1dif cells) was chosen. Dataset (raw expression dataset) GSE126831 for HCC was selected and processed for normalization and acquisition of log2 values from mapped genes which is well depicted by a workflow diagram shown in Figure 1a. GSE126831 encompasses samples (n=63) from day 0 to 10 (temporal samples infected with HCV and mocked samples). Whereas, samples (n=3) were for day zero (mocked RNA) and from day 1 to 10. There were three mocked and three RNA samples from cells infected with HCV(Lupberger *et al.,* 2019). Mocked samples were compared with HCV-infected samples for the respective day of infection for differential gene expression analysis. A detailed profile of mock mRNA and mRNA of HCV-infected cell lines was accomplished in an experimental design of a set of three. Profiling was performed on the data gathered every day between day zero and the 10^{th} day post-infection. Infection attained a plateau on the 7^{th} day observed post-infection (pi). An unspecific effect was observed even after the 7^{th} day post-infection that might not be omitted (Lupberger et al., 2019). All the samples (n=63) were processed for transcriptome profiling at Illumina NextSeq 500 (Homo sapiens) Paired-end reads generated from RNA sequence were aligned to reference human hg19 UCSC (The University of California Santa Cruz) reference using TopHat software (v2.0.14). The level of gene expression in terms of FPKM value (fragments per kilobase of exon model per million mapped reads) was determined using Cuffquant and Cuffnorm of the Cufflinks package (v2.2.1). Proteins and transcripts were precisely mapped in order to construct analysis groups. Genome build: hg19 Supplementary files format and content, Tab-delimited text files included RPKM values sample wise and results of a differential expression analysis of mapped transcripts. Gene expression patterns (Lapointe *et al.,* 2004; Subramanian *et al.,* 2005), its inferred functions (Mi *et al.,* 2015; Subramanian et al., 2005) and the impact of HCC bio-marker genes were comprehended. MATLAB functions (e.g. mattest) were applied so as to predict differential gene expression along with relevant statistical analyses. Kyoto Encyclopedia of Genes and Genomes (KEGG) database as well as the in-house code assigned to a specific pathway and a specific network analysis was applied to bring about pathway analysis delineation (Alexeyenko and Sonnhammer 2009)**.** FunCoup2.0 (Alexeyenko and Sonnhammer 2009) and Cytoscape (Okawa *et al.,* 2015) were applied for generating DEGs (differential expression genes) networks and for visualization of the network respectively. FunCoup is used for the prediction of 4 categories of functional associations for instance protein complexes, the interaction of the protein with another protein (protein-protein interaction) and various pathways (metabolic and signaling pathways) (Mi et al., 2015). MATLAB was used for most of the coding and calculation in the present study, moreover, many additional software and resources (PPI network database, ProgeneV2 and some other basic tools) were also used to achieve the goal of the study.

RESULTS

Known HCC bio-markers were collected from existing studies and the GEO dataset is a temporal (from day one--ten) dataset of mock and cells infected with HCV acquired to accomplish the study of the clinical relevance of HCC bio-markers, temporal gene expression profiling and functional relevance. Mapping out the percentage (%) of over-expression (both individual and overall) among the HCC samples and their co-occurrence for the selected and acquired genes bio-markers within TCGA database by using cBioPortal in HCC was carried out that have been presented in Figure 2. For co-occurrence, the network has also been presented with the respective inferred functions (pathways). Following the clinical significance of the over-expressed HCC bio-marker genes in the clinical specimen, a co-expression network

was drawn using co-relational values (as manifested in the network drawn on their corresponding edges) with the help of Cytoscape that has been shown in Figure 2c and it was observed that CCNB2, CLK2, CDK4, CDC7, E2F3, PCNA, MCM3, MCM4, USP1, KIF20A, MCM2, and MCM7 were predominantly regulating a greater number of genes or in other words, it could be said that most of the genes were dependent on each other.

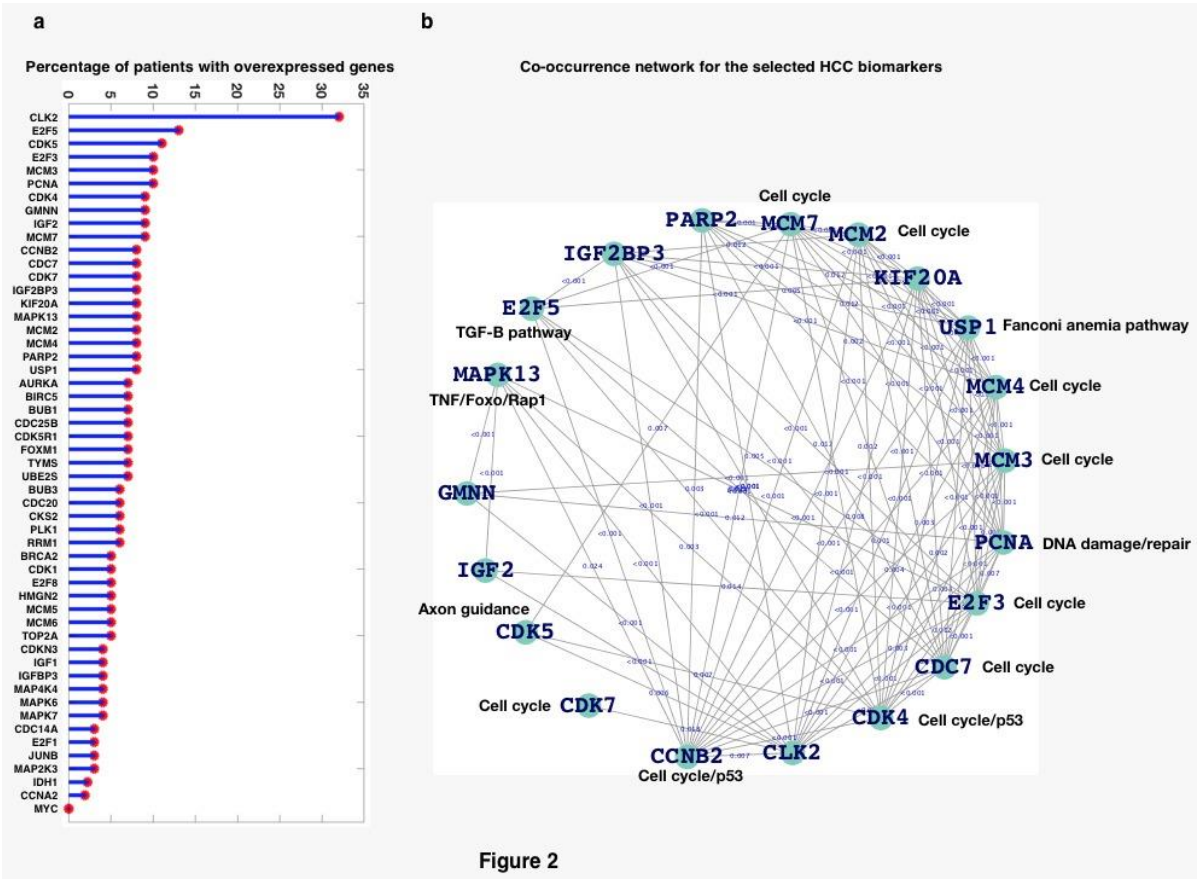

Fig. 2: Elucidation of over-expression and co-occurrence of significant HCC biomarkers. (a) Percentage of patients with over-expression for the respective genes (known HCC bio-markers), (b) Co-occurrence network for the selected HCC biomarkers.

Temporal Gene Expression Profiling of HCC As A Result of HCV Infection:

It was observed that all the selected HCC bio-markers were found to be clinically relevant to a significant level. The report of temporal gene expression profiling for HCC with and without HCV infection is laid down as shown with the help of Figure 3. Fluctuating of DEGs' number following an exponential increase in the number of DEGs was recorded up to day five (Figure 3a). In addition to that, a number of common DEGs (differentially expressed genes) between the overlapping steps were also mapped out. And it was ascertained that the exponential increase even in the count of common genes from one step to the next steps which from day five to eight appeared robust (Fig. 3b). Temporal gene expression profiling was further clarified by using of Venn diagram representing sets of genes categorized into two groups (group 1 with days 1—5 and group 2 with days 6—10). Venn diagram for DEGs list of group 1 and that for DEGs list of group 2 have been represented by Figure 3c and Figure 3d respectively. Temporal gene distribution with exclusive and common genes for the respective day of infection with HCV has been represented in Figure 3c and Figure 3d. It was determined

that during the early phase of infection, there were only a few genes that were common the next day of infection but with the increase in the time period of infection the number of common DEGs increased further and become stable in the last 3-4 days of infection phase.

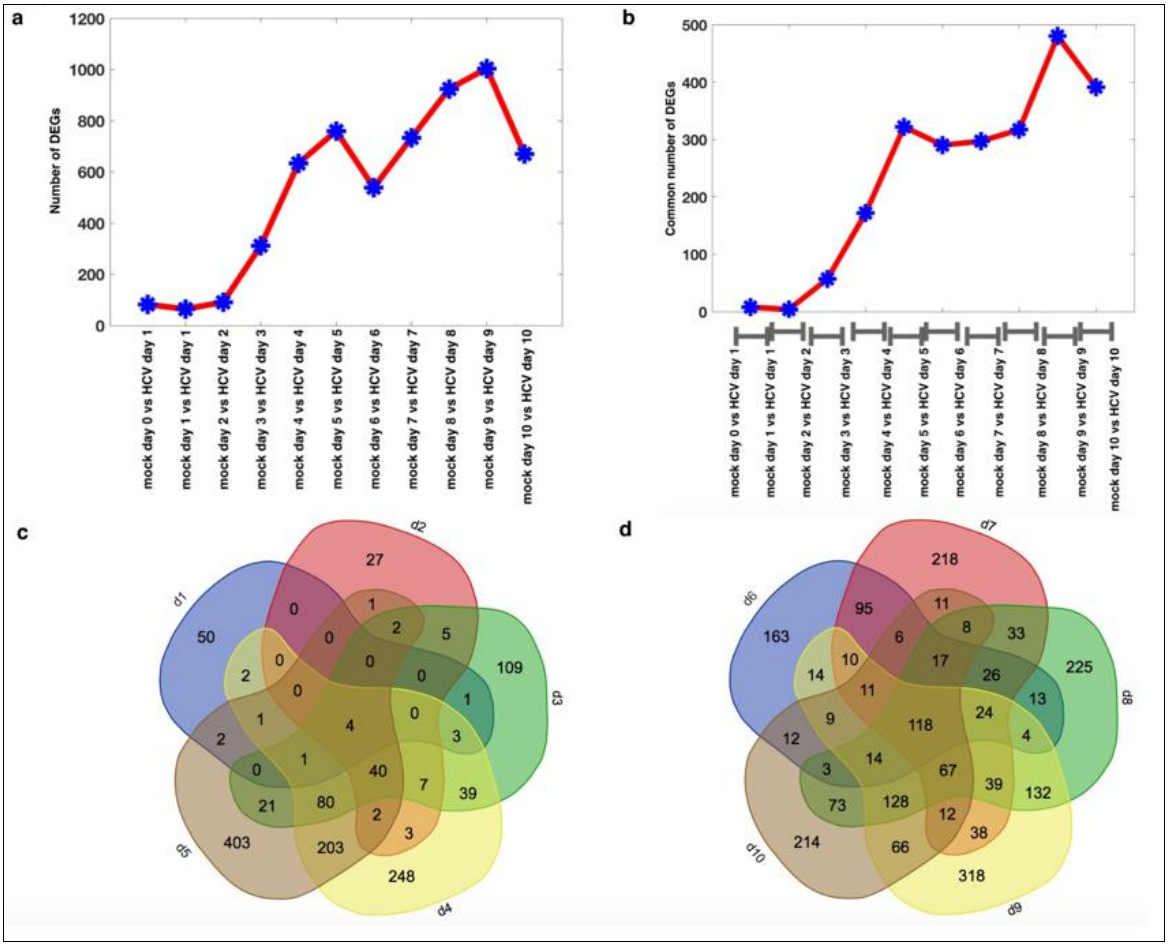

Fig. 3: Temporal gene expression profiling of HCC with HCV infection. (a) The number of DEGs from day one- day ten. (b) Number of common DEGs in different combinations (such as day one with day two, day two with day three, day three with day four, day four with day five, and so on). (c) Venn diagram represents the DEGs from day 1 (d1) to day 5 (d5). (d) Venn diagram represents the DEGs from day 6 (d6) to day 10 (d10).

Enriched Pathways Evolved Are Temporally Different With and Without HCV Infection In The Case Of HCC:

Pathway enrichment analyses for all the sets of DEGs list and prepared groups of enriched pathways were accomplished following gene expression profiling. There were two categories of the enriched pathway; group 1 and group 2 which encompass the list of enriched pathways from day three—six and that from day seven—ten respectively (Fig. 4).

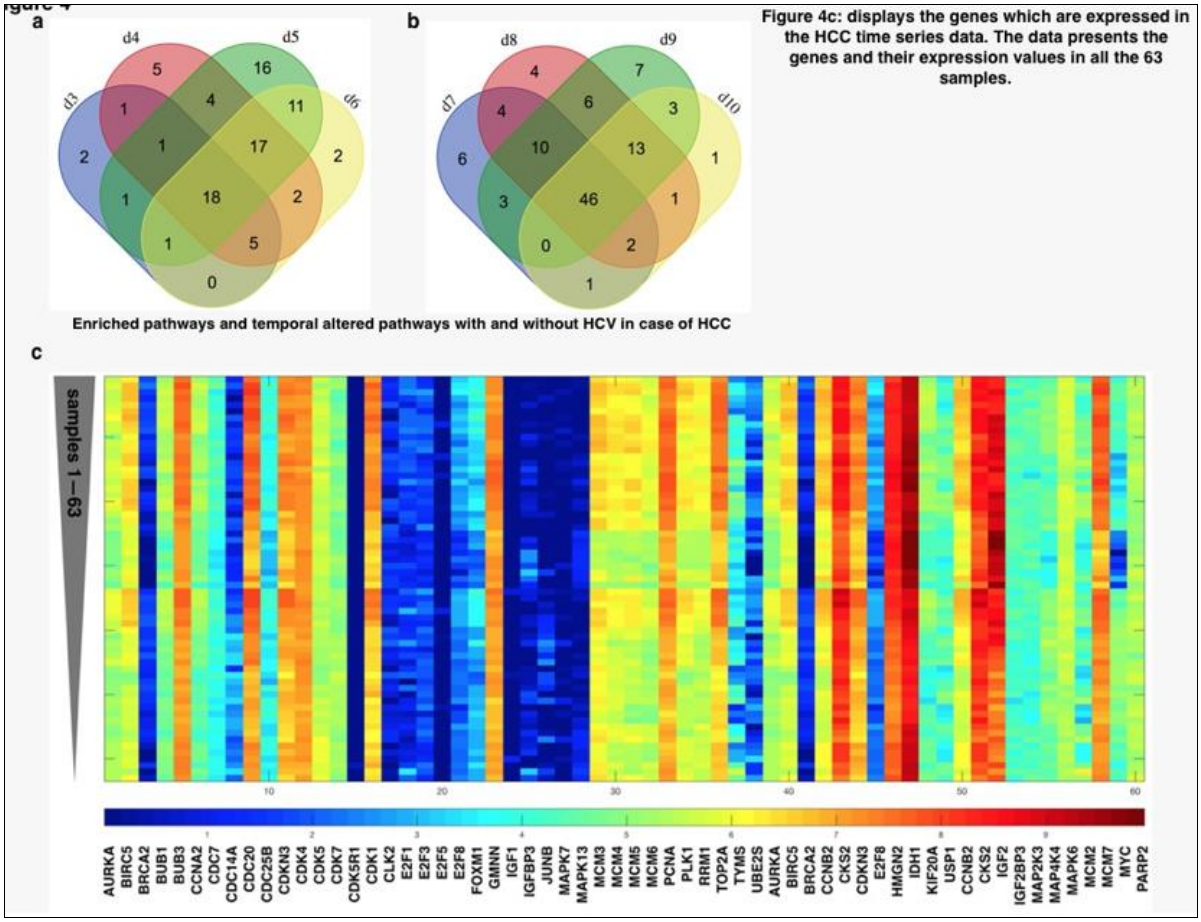

Fig. 4: Temporal alterations of functions in case of HCC due to HCV infection. (a) Venn diagram representing the enriched pathways from day 1 (d1) to day 5 (d5). (b) Venn diagram representing the enriched pathways from day 6 (d6) to day 10 (d10). (c) Displays the genes that are expressed in the HCC time series data. The data presents the genes and their expression values in all 63 samples.

The list of enriched pathways (day one and day two) for the Venn diagram was excluded because as there were only a few pathways (insignificant). Enriched pathway analyses evidently revealed that the shared enriched pathways appeared to be more robust and there were only a few exclusive pathways. The list of pathways and genes common between different groups have been presented for more clarity of the findings (Table 1 and Table 2 represent group 1 and group 2 respectively). The major difference between the temporal DEGs and enriched pathways list was that there were more pathways enriched with the passage of infection time. Among the most common enriched pathways were PI3K, cAMP, TGF, TNF, Rap1, Nf-kB, apoptosis, longevity regulating pathway, the interaction of cytokine-cytokine receptor, signaling pathways that regulate pleuripotency of stem cells, p53 protein signaling, Wnt signaling pathway, Hippo signaling pathways and Toll-like receptor signaling. The majority of these pathways have been well characterized for the immune-controlling system, infection and inflammation, and human diseases such as cancer.

Table 1. Enriched pathways from day 1 (d1) to day 5 (d5).

Names	Total	Pathways
Day 3-day 4 day 5 and day 6	18	PI3K-Akt/AKT signaling pathway and cAMP signaling pathway/adenylyl cyclase pathway
		Transforming growth factor-beta (TGF-beta) signaling pathway and osteoclast differentiation
		Tumor necrosis factor (TNF) signaling pathway and Rap1 signaling pathway
		NF-kappa B signaling pathway and MAPK signaling pathway
		cGMP-PKG pathway and apoptosis
		Longevity regulating pathway
		Pathways that regulate the pluripotency of the stem cells
		Interaction of cytokine and cytokine receptor and p53 pathway
		Wnt signaling pathway and Toll-like receptor (TLR) signaling pathway
		Hippo Signaling Pathway and Ras signaling pathway
Day 3-day 4-day 5	1	Apelin signaling pathway
Day 3-day 4-day 6	5	Neurotrophin signaling pathway, Adipocytokine signaling pathway
		T cell receptor signaling pathway and retinoic acid-inducible gene 1 (RIG-I)-like receptor signaling pathway
		B-cell receptor signaling pathway
Day 3-day 5-day 6	1	Phagosome
Day 4-day 5-day 6	17	Butanoate metabolism, PPAR signaling pathway, Cell cycle
		Glycerolipid metabolism, AMP-activated protein kinase (AMPK) signaling pathway and Peroxisome
		Glycine serine and threonine metabolism
		Retinol metabolism and drug metabolism by cytochrome P450
		The complement system and coagulation cascades
Day 3-day 4	1	Oxytocin signaling pathway
Day 3-day 5	1	Phospholipase D signaling pathway
Day 4-day 5	4	Cell adhesion molecules (CAMs) and Leukocyte transendothelial migration (LTM)
		Extracellular matrix (ECM)-receptor interaction and pathway for nitrogen metabolism
Day 4-day 6	2	NOD-like receptor (NLRs) signaling pathway
		Gonadotropin-releasing hormone (GnRH) signaling pathway
Day 5-day 6	11	Arginine metabolism, proline metabolism, Tyrosine metabolism
		Thyroid hormone (TH) synthesis, HIF-1 signaling pathway
		Valine, leucine as well as isoleucine degradation and pathway for pyrimidine metabolism
		Pathway for fatty acid metabolism and Jak-STAT pathway
		Primary bile acid biosynthesis, Tryptophan metabolism and Axon guidance
Day 3	2	Inflammatory mediator regulation of TRP channels
		Ubiquitin mediated proteolysis
Day 4	5	Adrenergic signaling in cardiomyocytes, Endocytosis
		Aminoacyl-tRNA biosynthesis, Fructose and mannose metabolism
		Ovarian steroidogenesis
Day 5	16	Sphingolipid signaling pathway, Glutathione metabolism
		Steroid biosynthesis and pathway for NK cell-mediated cytotoxicity
		Progesterone-mediated maturation of oocyte
		Tight junction
		Glycerophospholipid metabolism, Propanoate metabolism
Day 6	2	Contraction of vascular smooth muscle
		Interaction of neuroactive ligand-receptor

Table 2. Enriched pathways from day 6 (d6) to day 10 (d10).

Names	Total	Pathways
Day 10-day 7-day 8-day 9	46	Metabolic pathway of Arginine and proline and cell adhesion molecules (CAMs)
		PPAR signaling pathway, PI3K-Akt signaling pathway, Cell cycle
		cAMP signaling pathway, Tyrosine metabolism and Transforming growth factor beta (TGF-beta) pathway
		Adipocytokine pathway and signaling pathway of tumor necrosis factor (TNF)
		Glycerolipid metabolism and signaling pathway- AMPK
		Synthesis of Thyroid hormone and Peroxisome
		Metabolism of glycine serine and threonine
		Hypoxia inducible factor 1 (HIF-1) pathway and Rap1 pathway
		Process of leukocyte transendothelial migration, Phagosomal structure, Thyroid hormone signaling pathway
		MAPK, ECM, Apelin pathway, ECM-receptor interaction
		ErbB pathway and metabolic pathway of pyrimidine.
		Regulatory process of actin cytoskeleton and pathway of fatty acid metabolism
		Cyclic GMP-dependent protein kinase (cGMP-PKG) pathway, the process of focal adhesion and pathway for longevity regulation
		Pluripotency of stem cells and its regulatory signaling pathway
		Jak-STAT pathway, the process of primary bile acid biosynthesis and pathway for purine metabolism
		Interaction of cytokine with cytokine receptor
		The metabolic process of xenobiotics mediated by cytochrome P450 and retinol metabolic process
		Drug metabolic process - cytochrome P450 and interaction of the neuroactive ligand with its receptor
		Tryptophan metabolic process, complement pathway and coagulation cascades
		Hippo Signaling, Wnt pathway, Axon guidance, and Ras.
Day 7-day 8-day 9	10	Neurotrophin signaling pathway, Butanoate metabolism
		Osteoclast differentiation and Signaling pathways for phospholipase D
		NK cell-mediated cytotoxicity
		Valine leucine and isoleucine degradation and signaling pathway for estrogen
		Inflammatory mediator regulation of TRP channels
		Carbohydrate metabolism (starch and sucrose metabolic pathway), Alanine, aspartate and glutamate metabolic process
Day 10-day 7-day 8	2	Endocytosis, p53 signaling pathway
Day 10-day 8-day 9	13	Insulin signaling pathway, Glutathione metabolism
		Adrenergic signaling in cardiomyocytes and metabolic process of drug metabolism along with other enzymes
		Pyruvate metabolic process and Steroid hormone biosynthetic pathway
		Pathway for pentose phosphate metabolism, the metabolic process of amino sugar as well as nucleotide sugar
		Lysine degradation, metabolism of fructose and mannose sugars
		Calcium signaling pathway, Tight junction, Galactose metabolism
Day 7-day 8	4	Signaling pathway for T cell receptors and pathway for oxytocin signaling
		Prolactin pathway and pathway for GnRH signaling
Day 7-day 9	3	Pathway for NF-kappa B signaling mechanism, and pathway for retrograde endocannabinoid signaling
		Gap junction
Day 10-day 7	1	Protein processing in the endoplasmic reticulum
Day 8-day 9	6	Steroid biosynthesis, Nitrogen metabolism, Apoptosis
		Glycerophospholipid metabolism, Propanoate metabolism
		Ovarian steroidogenesis
Day 10-day 8	1	The metabolic pathway for porphyrin and chlorophyll
Day 10-day 9	3	Pathway of Notch signaling and N-Glycan biosynthesis, Adherens junction
Day 7	6	Circadian entrainment, Fc epsilon RI signaling pathway
		Signaling pathway of RIG-I-like receptor and process of Long-term depression
		Platelet activation, Toll-like receptor signaling pathway
d8	4	Sphingolipid signaling pathway, mTOR signaling pathway
		Progesterone-mediated oocyte maturation, Linoleic acid metabolism
d9	7	Synaptic vesicle cycle, Hematopoietic cell lineage, Aminoacyl-tRNA biosynthesis, Sphingolipid metabolism, Biosynthesis of unsaturated fatty acids
		Folate biosynthesis, Melanogenesis
d10	1	SNARE interactions in vesicular transport

DISCUSSION

Lethal hepatocellular carcinoma (HCC) constitutes seventy to eighty percent of all cases of liver cancer and contributes enormously to the cancer-associated mortality rate globally. Chronic and persistent HCV infection is one of the major etiologies of the hepato- carcinogenesis and development of HCC (Saito *et al.,* 1990). The absence of an HCV vaccine and limited options for therapeutic management of HCV-infected patients and its advancement to liver cirrhosis and then to HCC is quite alarming. Challenging beforehand diagnosis of HCV infection, lack of preventive and targeted therapy, as well as the absence of suitable markers for diagnosis, contribute enormously to high mortality rates in

patients suffering from HCV infection (Song *et al.,* 2016). Few studies have made an attempt to unravel and describe HCV-induced- HCC's molecular mechanisms (Lupberger *et al.,* 2019; McGivern and Lemon 2011; Wu *et al.,* 2019). A significantly high level of altered expression of host genes engaged in cell metabolism, internal cellular transport system and mechanism of defense at a cellular level has been demonstrated (Ishida *et al.,* 2011) and thus necessitating to explore (identification and characterization) vital cellular factors engaged in the replication cycle of HCV essential for the apprehension of pathogenesis and identification of definitive therapeutic target for HCV-induced-HCC. Clinical relevance of biomarkers in HCC, and determination of the significance of the over-expression of HCC biomarkers, DEGs and enriched pathways retrieved from the gene expression data were executed. The percentage of over-expression (both individually and overall) and co-occurrence for the selected genes (bio-markers) within The Cancer Genomic Atlas (TCGA) database were mapped out to delineate the clinical relevance of HCC biomarkers. It was found that all the selected HCC bio-markers were found clinically relevant as all these genes were assessed to be considerably over-expressed in all the clinical specimens under investigation which is corroborated by similar findings demonstrated in detail in many studies on the identification of cell proliferation knot (cluster) gene signatures in cases of HCC (Andrisani *et al.,* 2011). Co-expression network (protein-protein interaction) was drawn by using co-relational values and it was observed that a hub of genes such as CCNB2, CLK2, CDK4, CDC7, E2F3, PCNA, MCM3, MCM4, USP1, KIF20A, MCM2, and MCM7 was dominantly regulating a greater number of genes with a greater degree of connectivity and these hub genes could be considered as appropriate diagnostic and therapeutic targets for HCV-associated-HCC. Most of the genes of MCMs family (minichromosome maintenance) such as MCM2, MCM3, MCM4 and MCM7 have been found to be dominantly regulating the other HCC biomarkers (shows a high level of connectivity) which are considered as licensing factors for DNA replication process and remain involved in hepatocellular carcinogenesis (Liu *et al.,* 2018). One of the nodal genes in HCV-HCC is KIF20A (kinesin family member) whose over-expression might aggrandize proliferation as well as invasion of cancerous cells and thus play a vital role in hepatocellular carcinoma cells proliferation as demonstrated in many previous studies (Kawai *et al.,* 2018; Liu *et al.,* 2017; Shi *et al.,* 2016; Taniuchi *et al.,* 2014). Cyclin B2 (CCNB2) top nodal genes exhibiting higher grade of dominant connectivity which is corroborated by an investigation on progression, prognosis and expression in the case of HCC (Shan *et al.,* 2019). Mocked samples with HCV-infected samples for the respective day of infection were compared for differential gene expression analysis. Profiling of mRNA was performed on data gathered from either mock or HCV-infected Huh7.5.1dif cells (cell lines that support the enhanced production when allowed to be infected every day between zero-day and 10^{th}-day post-infection. Infection attained a plateau on the 7^{th} day observed post-infection (pi). The temporal gene expression profiling (expression level) for HCC with and without HCV infection unraveled a fluctuation in the number of DEGs following an exponential increase in the number of DEGs up to day five which signifies that during the early stage of infection of HCV in case of HCC, there is a great deal of distribution in gene expression pattern (Lupberger *et al.,* 2019). This finding of exponential increase in DEGs at the early phase of HCV infection is corroborated and authenticated by another study on level of co-expression assessment DEGs in HCV-induced-HCC (Song *et al.,* 2015). Following the mapping of a number of common DEGs between the overlapping steps, an exponential increase even in the number of common genes from one step to the next

steps was ascertained while from day 5 to day 8 it appeared robust which suggests that there is a set of genes stays differentially expressed after first few days (Grimes *et al.,* 2013; Song *et al.,* 2015). Temporal gene expression profiling was elucidated by using Venn diagrams depicting (Figure 3c and 3d) sets of genes categorized into two groups (group 1 with day 1—5 and group 2 with day 6—10) and revealing temporal gene distribution with exclusive and common genes for the respective day of infection with HCV. Presence of only a few genes which were common with next day of infection but with the increase in time period of infection the further increment in the number of common DEGs and their eventual stability in the last phase (3-4 days) of infection is in accordance with stability followed by fluctuation in the level of gene expression demonstrated in a previous investigation (Ahmad *et al.,* 2012; Song *et al.,* 2015) that suggests that the first few days of infection phase are more prone to develop HCC. At some point of time during infection state the reduction in the expression level of certain host genes which has also been determined in one study which describes the decrement in the expression of genes engaged in lipid metabolism, the process of transport within the cell renders decreased viral multiplication and release suggesting the possible significance of these genes in regulating the replication cycle of HCV (Zeisel *et al.,* 2011). Enriched pathway analyses for all the sets of DEGs list and prepared groups of enriched pathways (There were two categories of enriched pathway group 1 and group 2 which encompasses the list of enriched pathways from day three—six and that from day seven—ten respectively) evidently revealed the shared enriched pathways had appeared to be more robust and there were only a few exclusive pathways. The major difference between the temporal DEGs and enriched pathways list was that there was more number of pathways enriched with the passage of infection time which reflects that the chronicity of HCV infection plays a critically vital role in eventuating HCC. Although temporal (time-dependent) gene-expression pattern has been analyzed in many previous studies (Grimes *et al.,* 2013; Zeisel *et al.,* 2011), however the findings of the present investigation which is the functional impact of a cluster of hub genes from day one of infection to day 10 in combination with clinically significant known HCC-biomarkers would serve in terms of the more comprehensive approach of diagnosis and therapy of HCV-induced-HCC.

Conclusion

Present analyses elucidate the altered pathways in the case of HCC infected with HCV related to the immune control system, inflammatory process and tumorigenesis. 12 hub genes were identified to be the biomarkers for diagnosis and therapeutic target for clinical management of HCV- associated-HCC. Various i*n-vitro* and *in-vivo* detailed gene expression investigations are recommended to validate the findings.

Author contribution:

Author Shaia Saleh R. Almalki has made the sole contribution in executing analyzing, writing and editing the manuscript.

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Conflict of Interest:

The author declares no conflicts of interest.

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