RESEARCH ARTICLE

Molecular Prognostic Profile of Egyptian HCC Cases Infected with Hepatitis C Virus

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Abstract

Background: Hepatocellular carcinoma (HCC) is a common and aggressive malignancy. Despite of the improvements in its treatment, HCC prognosis remains poor due to its recurrence after resection. This study provides complete genetic profile for Egyptian HCC. Genome-wide analyses were performed to identify the predictive signatures. Patients and Methods: Liver tissue was collected from 31 patients with diagnosis of HCC and gene expression levels in the tumours and their adjacent non-neoplastic tissues samples were studied by analyzing changes by microarray then correlate these with the clinico-pathological parameters. Genes were validated in an independent set by qPCR. The genomic profile was associated with genetic disorders and cancer focused on gene expression, cell cycle and cell death. Molecular profile analysis revealed cell cycle progression and arrest at G2/M, but progression to mitosis; unregulated DNA damage check-points, and apoptosis. Result: Nine hundred fifty eight transcripts out of the 25,000 studied cDNAs were differentially expressed; 503 were up-regulated and 455 were down-regulated. A total of 19 pathways were up-regulated through 27 genes and 13 pathways were down-regulated through 19 genes. Thirty-seven genes showed significant differences in their expression between HCC cases with high and low Alpha Feto Protein (AFP ≥600 IU/ml). The validation for the microarray was done by real time PCR assay in which *PPP3CA, ATG-5, BACE* genes showed down-regulation and *ABCG2, RXRA, ELOVL2, CXR3* genes showed up-regulation. cDNA microarrays showed that among the major upregulated genes in HCC are sets. Conclusion: The identified genes could provide a panel of new diagnostic and prognostic aids for HCC.

Keywords: Egyptian HCC - hepatitis C virus - gene expression profile - microarrays

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Introduction

Hepatocellular carcinoma (HCC) is a global health problem being a most common cause of cancer-related death and accounts for 80-90% of primary liver cancer. Early diagnosis is uncommom and medical treatments are inadequate (Altekruse et al., 2009). HCC incidence is increasing dramatically, with marked variations among geographic areas (El-Serag and Rudolph, 2007) and environmental risk factors (London et al., 1995; Yu and Yuan, 2004). Most HCC cases occur in either sub-Saharan Africa or in Eastern Asia (Castello et al., 2010) and is the most threatening malignancies in Egypt, and counts 4.7% of chronic liver disease patients with rate increasing (Anwar et al., 2008). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major cause of liver disease worldwide and may contribute directly to the genesis of HCC. In Europe and America, chronic HCV and alcoholic cirrhosis are the main risk factors for HCC. Among patients with HCV and cirrhosis, the annual incidence rate of HCC ranges between 1-8%, being higher in Japan (4-8%), intermediate in Italy (2-4%) and lower in USA (1.4%) (Fassio, 2010). HCV infection is high in Egyptian and its percentage in patients who develop HCC is higher than that of any other countries.

Development and progression of HCV-induced HCC involves cascade of genetic alterations but details still unknown. Genomic-based approaches using oligonucleotide microarrays represent reliable technology of choice. Identification and monitoring of gene expression profile changes in HCC tissues may provide chance to identify novel targets for disease detection and intervention. We aim to study the mechanism of hepatocarcinogenesis by investigating the gene expression

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profile in Egyptian patients infected with HCV.

Materials and Methods

Patients and tissue samples collection

The study included 31 HCC patients attended the National Cancer Institute, Cairo University. Demographic and clinicopathological characteristics of HCV-cirrhotic patient groups collected from patients' records were summarized in Table 1. The study was conducted in compliance with Helsinki Declaration and was approved by the senior staff committee. Tumors, their adjacent non-neoplastic tissues (ANT) and blood samples were obtained. Tissues were cut into two parts, the first part was used for routine histolopathological examination and the second was immediately snap-frozen for RNA and DNA extraction.

Serological markers

Serological markers for HBV and HCV infections were detected with the current standard assays (enzyme immunoassay [EIA]; Innogenetics, Belgium) according to manufacturer's instructions.

Detection of HCV-RNA

Viral RNA was extracted from patients' sera according to manufacturer's instructions by Qiagen (Germany). The RT-PCR was performed by real time PCR (Applied biosystem, life technology, USA).

Detection of occult HBV DNA

DNA was extracted from frozen liver tissues according to standard protocol and was analyzed for HBV genomes with polymerase chain reaction (PCR) assays to detect the S, X and core genes as previously described (Kao et al., 2002) to exclude occult HBV infection.

cDNA microarray

Total RNA was isolated using Trizol (Invitrogen, Germany) followed by RNeasy Mini Kit (Qiagen, Germany). All isolated RNA samples followed and met purity and integrity for the quality control criteria previously established in our laboratory (Zekri et al., 2008). Reactions for cDNA synthesis microarray hybridization, image generation, and probesets reading process were performed as reported previously (Zekri et al., 2008). In brief, two microgram of RNA from tumours and ANT were reverse transcripted and labelled with Cy3 and Cy5 (Amersham Biosciences, UK) following guidelines and recommendations. Each sample was tested in triplicate on array 40K supplied from Fox Chase Cancer Center. Labelled cDNA from tumor and ANT were hybridized onto a cDNA chip containing the entire human genome according to the posted protocol http://www.fcc.edu/rsearch/facilities/biotechnology/DNAMicroarray.htm. The resulting microarray dataset in this paper including information about the experiment and array design was packaged according to the MAGE-TAB standard format and deposited at the ArrayExpress repository (www.ebi.ac.uk/arrayexpress) with the ID E-MTAB-326 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-326)"

Table 1. Clinical Features of the Studied Patients

Variable		HCC 31 (%)
Gender:	Male	20 (75%)
	Female	11 (25%)
AST (IU/ml)		64.96±28.03
ALT (IU/ml)		49.9±18.54
AFP (IU/ml)		1337.43±2112.85
HCV-PCR		31/3(100%)
HBV		0 (0)
Tumor site:	Right	20 (65%)
	Left	11 (35%)
Tumor size:	≤2cm	20 (65%)
	>2cm	11 (35%)
Grade:	I+II	24 (77%)
	III	7 (23%)
Safety margin:	Pos	9 (29%)

Microarray quality control and data analysis

Quality of the hybridized arrays was assessed by examining the average background, scaling factor, percent of probe sets called present by the detection call algorithm, and the 3':5' ratio for endogenous housekeeping genes present on the chips. Additionally, probe level linear models were fit and plots of the residuals were examined for each hybridized Gene Chip. Normalized and log-transformed expression data served as input for significance analysis of microarray with standard parameters. The hierarchical clustering method (using web-available software "Cluster", "Tree View" and Genesis software) were applied to both genes and samples by using Pearson r test (Zekri et al., 2000). The hierarchical clustering algorithm was applied to all genes. Information about genes participating in different function was obtained from Onto-Express Soft http://vortex.cs.wayne.edu. Information about genes participating in known signaling pathways was derived from Entrez Gene http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene and KEGG pathway http://www.genome.jp/kegg/pathway.html databases. To identify members of particular pathways, we combined KEGG gene number with the identifier/accession number.

For gene expression analysis, Mann Witney Test was used for numeric variables, and Chi square or Fisher's exact Test was used to analyze categorical variables (p value was considered significant when $P \leq 0.05$). We used Scan Array Express II (Perkin Elmer, USA) software for image processing. Measured intensities were analyzed using the Genesis software and R program that detect the up- and downregulated genes according to the ratio in their software's.

Validation of micrroarray results by Real Time PCR assays

The remaining total RNA extracts, not used in the microarray studies, was used for the study the expression of the selected genes RXRA, ELOVL2, CXCR3, PPP3CA, ATG5, BACE using real-time PCR. The RNA was reverse-transcribed to cDNA using oligo (dT) primers and high capacity cDNA kit. Taqman Gene Expression Assay reagents (Applied Biosystems, CA, USA) were employed in real-time PCR using Applied Biosystems

7,500 Real-Time PCR System. The PCR thermal cycling conditions were an initial step of 20 sec at 95°C, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Quantification of the target genes was based on the relative standard curve method and was normalized to the expression of GAPDH encoding gene as internal standard as its ratio between the tumor and ANT tissues was the one closest to unity (0.98).

Results

Patients, tumor subtypes and characteristics

HCC and their adjacent non-neoplastic tissues were investigated for gene expression profiling. Table 1 showed tumor grade, size and demographic data of HCC patients. There were no significant relationships between HCC and age. There were statistically significant different between HCC with the mean size of tumor and the mean number of histological grade and cirrhosis.

Demographically, more than 50% of patients were above 60 year old with mean age at diagnosis 58±9.6 years, ranging between 46 and 72 year old. No statistical differences were found regarding gender. HCC patients had mean±SD AST of 64.96±28.03 U/dL, ALT of 49.9±18.54 U/dL and AFP was 1337.43±211 IU/ml. All patients were HCV infected and none of them were positive for HBsAg. Out of the 31 HCC patients, 65% had tumor size ≥2 cm and 77% had tumor grade I or II and 23% had grade III Table 1.

Table 2. The Correlation between the Expression Level of the Studied Genes and Clinicopathological Features of Hepatocellular Carcinoma Cases

		PPP3CA	ATG	BACE1	ABCG2	RXRA	ELOVL2
Tumor size:	≤2cm	71.40%	93.00%	60.70%	93.00%	78.60%	64.30%
	>2cm	83.00%	66.70%	66.70%	83.00%	83.00%	75.00%
Grade:	I+II	72.00%	84.40%	56.30%	87.50%	78.00%	62.50%
	III	87.50%	87.50%	87.50%	100%	87.50%	87.50%
Cirrhosis:	Present	83.00%	96.60%	69.00%	93.00%	79.00%	62.00%
	Absent	55.00%	55.00%	46.00%	82.00%	82.00%	72.00%
CAH:	Pos	77.00%	82.00%	68.00%	0	82.00%	73.00%
	Neg	72.00%	89.00%	55.60%	72.00%	77.80%	61.00%

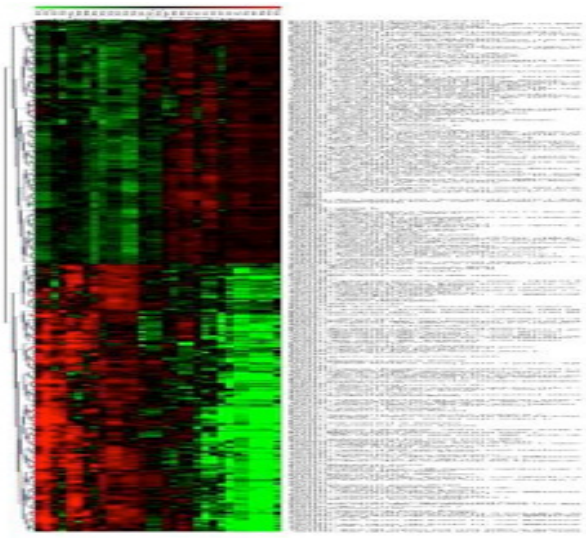

Figure 1. Hierarchical Cluster of the Differentially Expressed Genes in Egyptian HCC Associated with HCV Infection. Green color indicates lower expression, while red color indicates higher expression

We conducted three independent microarray assays starting from independent mRNA isolations and defined differential expression based on their consensus. From the analysis, total of 958 genes/transcripts were significantly and differentially expressed in HCC cases, 503 showed up regulation and 455 showed down regulation. Hierarchical clustering of the differntially expressed genes is shown in Figure 1.

Functional classification of genes with distinct expression in HCC

Gene ontology and molecular pathways identified the associated network functions related to cellular assembly and organization; DNA replication, recombination, and repair; genetic disorders, hematological disease, and cancer; and cell cycle control and cell morphology. To understand how the distinctly expressed genes were involved in the different pathways, we analyzed the gene ontology and functional annotation using the Database for Annotation. In addition, we searched the GO Ontology database, as well as the PubMed data-base, for each gene to identify the functions of genes then grouped the genes.

Gene ontology and pathway analysis of 958 genes revealed that 285 genes from the 503 up-regulated genes

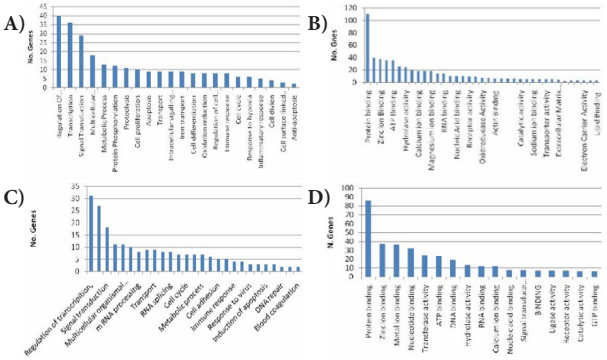

Figure 2. Up and Down Regulated HCC Genes involved. A (up) and C (down) Biological Process, B (up) and D (down) Molecular Function

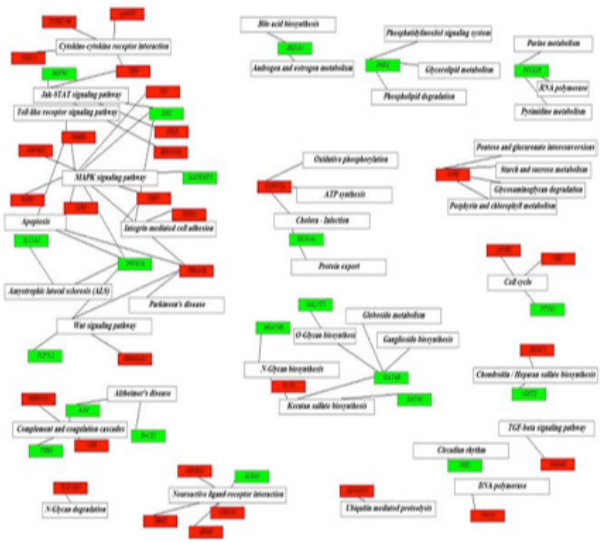

Figure 3. The Result of the Whol Pathway Scope. The Interplay between the Involved Genes and Pathways (Red is for Up-regulated Genes and Green for Down-regulated)

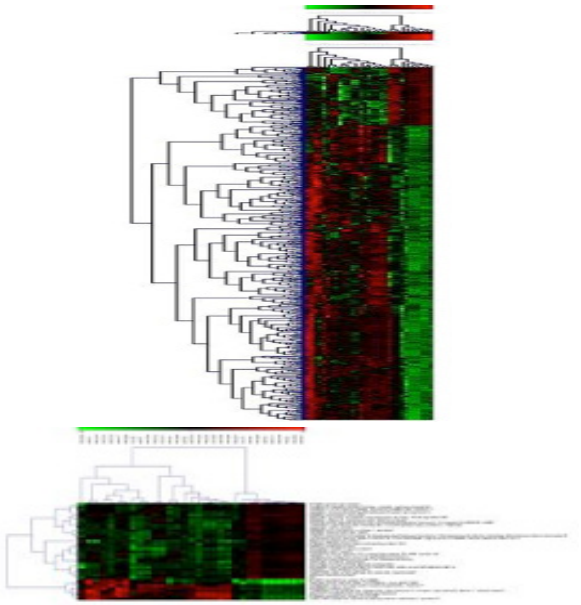

Figure 4. Hierarchical Clustering of the Genes That Showed Differential Expression on with Respect to AFP. Left: Cluster of the genes that passed the t-test, where the samples with elevated AFP are grouped together on the right side. Right: Cluster of the samples that further passed the verification step; the samples with elevated AFTP are grouped together on the right side, as well

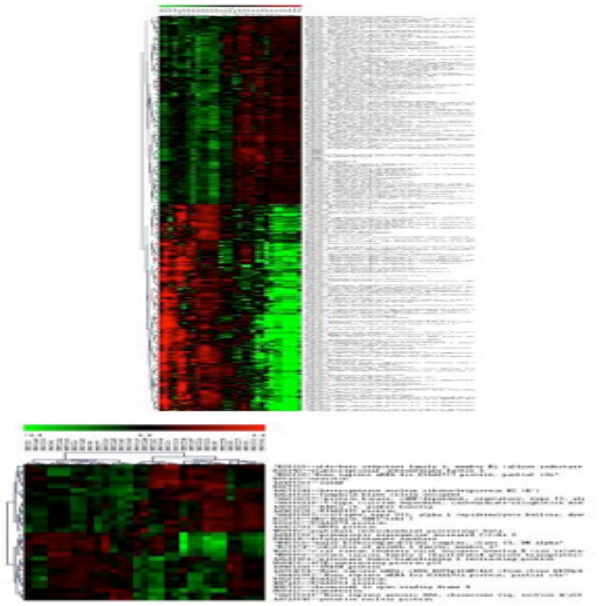

Figure 5. Hierarchical Clustering of the Genes That Showed Differential Expression on with Respect to Cirrohsis and Chronic Hepatitis. Left: Cluster of the genes that passed the t-test in case of Cirrohosis, where the samples with Cirrohosis are grouped together on the right side. Right: Cluster of the genes that passed the t-test in case of Chronic Hepatitis, where the samples with Cirrohosis are grouped together on the right side

are involved in biological processes (such as transcription, signal, transcription, signal transduction) (Figure 2), 289 in molecular function (Figure 3), and 310 in cellular components. Fifty-eight genes are involved in 65 different pathways. The most frequently detected genes in pathways are *v-raf-1* in 25; *PRKACB* and *IKBKG* in 13; *PPP3R1* in 10; *PDPK1* in 7; *Cas-Br-M* (murine) in 6; *RPS6KB1, RXRA* and *FGFR1* in 5; *WNT2* and *LAMA3* in 4 pathways.

From the 455 down-regulated genes, 210 are involved in biological processes (Figure 2), 233 in molecular function (Figure 2), 240 in cellular components and 49 are involved in 59 pathways (Additional file 4). *SOS1* in 20 pathways; *PPP3CA* in 11 pathways, *TCF7L2* in 9 pathways, *ITGAV* in 5 pathways and *WASL* in 4 pathways; *LTA* in 3 pathways, *ARHGEF2; ST3GAL2, ETS1; ST3GAL4* and phosphorylase kinase, alpha 2 (liver) (*PHKA2*) in two pathways. The most characteristic genes related to virus response were; virus-induced signaling adapter *VISA, ISGF3G* and *FGR*.

On the other hand, 47 pathways contained some genes that are up regulated and some that are genes down regulated. While 19 pathways only were specifically up regulated by 26 genes, 13 pathways were specifically down regulated by 19 genes. Figure 3 showed pathways' summary and the relationship between the involved genes and pathways (Red is for up-regulated genes and Green for down-regulated) as visualized by *WholePathwayScope* (http://wholepathwayscope.software.informer.com) software tool.

Figure 4 showed hierarchical clustering to figure out differentially expressed genes between samples with and without elevated AFP level (≥600 IU/ml).

Cirrohsis and chronic hepatitis: Figures 5 showed the genes that are differentitally expressed between HCC patients with and without cirrhosis and between HCC patients with and without chronic hepatitis e.g Interferon gamma receptor 1 (IFNGR1), Plasminogen-like B2 (PLGLB2), Coagulation factor II (thrombin) (F2), Tumor-associated calcium signal transducer 2 (TACSTD2) and Metastasis associated 1(MTA1) genes.

Discussion

Hepatocellular carcinoma is associated with advanced cirrhosis and most occurrs in patients with known risk factor such as chronic liver inflammation and cirrhosis (Altekruse et al., 2009). HBV and HCV viruses are the major cause of liver disease worldwide in which the incidence of HCV infection is hard to quantify since it is often asymptomatic and no active or passive vaccination is available yet. In HCV-cirrhotic patients, HCC is the final result of several rounds of hepatocytes destruction and regeneration by virus-induced immune-mediated mechanism and tissue damage (McGivern and Lemon, 2011; Yamashita et al., 2011).

The World Health Organization estimates that 3% of world's population are chronically HCV-infected and are currently at elevated risk for HCC (Ryder, 2003). Progressive liver damage until HCC development in chronic HCV-infected patients encompasses deregulation of molecular pathways triggering malignant transformation.Therfore the altered genes expression may help in diagnosis and therapeutic targets in HCC patients. The current study showed that out of the 31 HCCs cases, 958 transcripts were differentially expressed, 503 showed up-regulation and 455 showed down-regulation. We compared up- and down-regulated genes identified by the

cDNA microarray assays in accordance with their potential molecular functions, implicated in biological processes and sub-cellular localization.

The current data is in concordant with that of Ciccaglione et al (2008) who identified new genes that revealed regulation by HCV of new biological processes like chromatin packaging and chromosome segregation those different from our study this may be due to the fact that they used cell lines only whereas our study was based on human samples (Ciccaglione et al., 2008). Out of the 503 up-regulated genes, 285 genes are involved in biological processes, 289 in molecular function, and 310 in cellular components. Up-regulated genes had a strong association with the regulation of cell cycle progression, transcription, nucleic acid metabolism, and protein metabolism. In addition, genes related to cell responses, external growth stimuli, signal transduction, cell morphogenesis and biogenesis were more frequently found in the HCC up-regulated genes. The HCC up-regulated genes had more genes with their products distributed in cell nucleus, while the HCC down-regulated genes had more genes of secreted proteins. The most frequently detected genes in pathways are *v-raf-1* in 25; *PRKACB* and *IKBKG* in 13; *PPP3R1* in 10; *PDPK1* in 7; *Cas-Br-M* (murine) in 6; *RPS6KB1*, *RXRA* and *FGFR1* in 5; *WNT2* and *LAMA3* in 4 pathways.

The *RXRA*/*PPARr* is a member of nuclear receptor superfamily of transcription factors. The *RXRA* and *IKBKG* genes, which regulate Adipocytokine signaling pathway, were up-regulated in our study. Wang et al showed that the liver-enriched transcription factors *RXRa*/*PPARa* can support HBV transcription and replication in nonhepatic cells, indicating that liver-specific gene transcription is one of the determinants of HBV hepatotropism and could also do the same function in HCV infection (Wang et al., 2006).

From the 455 down-regulated genes, 210 were involved in biological processes, 233 in molecular function, 240 in cellular components and 49 were involved in 59 pathways.

The most downregulated genes are *SOS1* in 20 pathways; *PPP3CA* in 11 pathways, *TCF7L2* in 9 pathways, *ITGAV* in 5 pathways and *WASL* in 4 pathways; LTA in 3 pathways, *ARHGEF2; ST3GAL2, ETS1; ST3GAL4* and phosphorylase kinase, alpha 2 (liver) (*PHKA2*) in two pathways. The most characteristic genes related to virus response were; virus-induced signaling adapter *VISA, ISGF3G* and *FGR*.

The ABCG2, ATP-binding transmembrane proteins, plays an important role in drug resistance. In HCC the role of ABCG2 is closely associated with 'side population (SP)' a minor subset of cancer stem-like cells with unique capacity. In the current study ABCG2 genes was up-regulated. Similarly, ABCG2 is expressed in HCC tissues with significant expression pattern that influenced the levels of drug efflux from HCC cell lines (Hu et al., 2008). They verified the protective role of intrinsic ABCG2 expression in HCC cells by modulation of the SP proportion to the Akt signaling inhibitors through altering the subcellular localization of ABCG2 transporter. Zen (2007) found that ABCG gene was scattered or focally clustered in HCC. The overexpression of ABCG2 gene in the Egyptian HCC cases clarified the Akt high expression levels described in similar cases studied by our group (Kao et al., 2002).

ELOVL2 gene is involved in oxidative stress induction and lipid biosynthesis and is responsible for the elongation of long chain fatty acids. HCV infection induces modifications in lipid metabolism and contributes to the development of oxidative stress (Su et al., 2002; Chang et al., 2008). Recent reports revealed that the increased in the synthesis of fatty acids enhances HCV replication (Kapadia and Chisari, 2005). Our results showed upregulation of *ELOVL2* gene and is in agreement with Elam et al. (2009) who reported *ELOVL2* overexpression in morbidly obese women.

The products of up-regulated genes are mainly located in the nucleus, while the products of down-regulated genes are mainly secretory proteins. The current study showed that *BACE1* gene was overexpressed in 65% of our cases. *BACE1* gene is related to hepatic encephalopathy and may play an important role in cross talk between liver and brain. Prasanthi et al. (2009) proved that *BACE1* is probably responsible for inducing fatty liver (Prasanthi et al., 2009).

CXCR3-associated chemokines are important candidates for development of hepatic necroinflammation and fibrosis in chronic HCV infection. The intrahepatic mRNA levels of IFN-inducible T cell alpha chemoattractant (I-TAC), another *CXCR3* chemokine ligand, correlate with both portal and lobular inflammation. Several investigators found that the intrahepatic *CXCL10*/*IP-10* mRNA expression was associated with increased lobular necroinflammation (Harvey et al., 2003; Zekri et al., 2009). Our results showed overexpression of *CXCR3* gene in 75% of HCC cases.

ATG5 contributes to autophagic cell death by interacting with *Fas-associated* protein. Impaired autophagy in liver tissue causes accumulation of wasted organelles, leading to hepatomegaly. The HCC downregulated genes related to loss of normal physiological function of the hepatocytes. Among the down regulated genes in the current study is *ATG5* in 65% of the studied HCC cases. Down-regulation of *ATG5* expression in HeLa cells suppresses cell death and vacuole formation induced by IFN-gamma (Singh et al., 2009).

The knockdown of *PPP3CA* protein enhances up-regulation of *VEGF, FGF2, MAPK3* and *AKT1* (Wang et al., 2008) which is in agreement with this study that showed down regulation of *PPP3CA* in 75% of the studied cases and up regulation of this genes as well as our previous study (Zekri et al., 2008).

In conclusions, the present study provided the genomic profile associated with Egyptian HCC HCV-infected livers, also corroborating in part our previous priliminary study. More importantly, we identified numerous genes that associated with liver carcinoma which is related to clinical features of HCC. The most detected up-regulated genes are *v-raf-1, PRKACB and IKBKG, PPP3R1, PDPK1, Cas-Br-M* (murine), *RPS6KB1, RXRA* and *FGFR1, WNT2* and *LAMA3*. The down-regulated genes are *SOS1, PPP3CA, TCF7L2, ITGAV* and *WASL, LTA, ARHGEF2, ST3GAL2, ETS1, ST3GAL4* and *PHKA2*. The most characteristic genes related to virus response were;

virus-induced signaling adapter *VISA*, *ISGF3G* and *FGR*. Further analyses in HCC patients are required.

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