Molecular Signatures Associated with HCV-Induced Hepatocellular Carcinoma and Liver Metastasis

Valeria De Giorgi2*, Luigi Buonaguro1, Andrea Worschech3, Maria Lina Tornesello1, Francesco Izzo4, Francesco M. Marincola2, Ena Wang2, Franco M. Buonaguro1*

1 Molecular Biology and Viral Oncogenesis and AIDS Refer. Center, Ist. Naz. Tumori “Fond. G. Pascale”, Naples, Italy, 2 Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center and Trans-NIH Center for Human Immunology (CHI), National Institutes of Health, Bethesda, Maryland, United States of America, 3 Department of Biochemistry, Biocenter, University of Wuerzburg, Am Hubland, Wuerzburg, Germany, 4 Hepato-biliary Surgery Department, Ist. Naz. Tumori “Fond. G. Pascale”, Naples, Italy

Abstract

Hepatocellular carcinomas (HCCs) are a heterogeneous group of tumors that differ in risk factors and genetic alterations. In Italy, particularly Southern Italy, chronic hepatitis C virus (HCV) infection represents the main cause of HCC. Using high-density oligoarrays, we identified consistent differences in gene-expression between HCC and normal liver tissue. Expression patterns in HCC were also readily distinguishable from those associated with liver metastases. To characterize molecular events relevant to hepatocarcinogenesis and identify biomarkers for early HCC detection, gene expression profiling of 71 liver biopsies from HCV-related primary HCC and corresponding HCV-positive non-HCC hepatic tissue, as well as gastrointestinal metastases paired with the apparently normal peri-tumoral liver tissue, were compared to 6 liver biopsies from healthy individuals. Characteristic gene signatures were identified when normal tissue was compared with HCV-related primary HCC, corresponding HCV-positive non-HCC as well as gastrointestinal liver metastases. Pathway analysis classified the cellular and biological functions of the genes differentially expressed as related to regulation of gene expression and post-translational modification in HCV-related primary HCC; cellular Growth and Proliferation, and Cell-To-Cell Signaling and Interaction in HCV-related non HCC samples; Cellular Growth and Proliferation and Cell Cycle in metastasis. Also characteristic gene signatures were identified of HCV-HCC progression for early HCC diagnosis.

Conclusions: A diagnostic molecular signature complementing conventional pathologic assessment was identified.

Citation: De Giorgi V, Buonaguro L, Worschech A, Tornesello ML, Izzo F, et al. (2013) Molecular Signatures Associated with HCV-Induced Hepatocellular Carcinoma and Liver Metastasis. PLoS ONE 8(2): e56153. doi:10.1371/journal.pone.0056153

Editor: Niyaz Ahmed, University of Hyderabad, India

Received: June 18, 2012; Accepted: January 7, 2013; Published: February 18, 2013

Copyright: © 2013 De Giorgi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Italian Ministry of Health - Ministero Italiano Salute (Ricerca Corrente 2010–11; Programma Ricerca Oncologica Grant n° ACC4/2007–2012; Programma Integrato Oncologia Grant n° RO 4/2007 (2009–2012). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: irrccsvir@unina.it (FMB); degiorgiv@mail.nih.gov (VDG)

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death in the world [1–3]. As for other cancers, the etiology of HCC is multifactorial and progresses through multiple stages [4]. This multistep process may be divided into chronic liver injury, inflammation, cell death, cirrhosis, regeneration, DNA damage, dysplasia and finally HCC. Different lesions have been considered pre-neoplastic in regard to the development of HCC. For instance, cirrhotic liver contains regenerative nodules and like HCC may contain dysplastic nodules [5,6]. The principal risk factor for the development of HCC is hepatitis B virus (HBV) [7,8], followed by hepatitis C virus (HCV) infection [9]. Non viral causes are less frequent and include toxins and drugs (e.g., alcohol, aflatoxins, microcytosis, anabolic steroids), metabolic liver diseases (e.g., hereditary haemochromatosis, α1-antitrypsin deficiency), steatosis [10] and non-alcoholic fatty liver disease [11,12]. In general, HCCs are more prevalent in men than in women and the incidence increases with age.

The molecular mechanism underlying HCC is currently unknown. Activation of cellular oncogenes, inactivation of tumor suppressor genes, over-expression of growth factors, possibly telomerase activation and DNA mismatch repair defects may contribute to the development of HCC. Alterations in gene expression patterns accompanying different stages of growth, disease initiation, cell cycle progression, and responses to environmental stimuli provide important clues to these complex process [13,14]. In addition to primary HCC, metastatic liver disease often occurs. Metastases most often derive from gastrointestinal organs, primarily colon and rectum, though they can occur from primaries throughout the body [15]. These cancers can be treated using routine therapies relevant to the primary such as chemotherapy, radiotherapy, surgical resection, liver transplantation, chemo-embolization, cryosurgery or combination therapy [16]. The characterization of genes that are differentially expressed during tumorigenesis is an important step toward the identification of the biological steps involved in the transformation process. Studies examining the gene expression of metastatic liver tumors and HCC in parallel with paired non-cancerous liver tissues might yield important insights by identifying genes not expressed in normal liver and are switched on in tumors and vice versa. Such studies should also lead to
the identification of genes that are expressed in tumors at different stages and never in non-cancerous liver tissue.

The present study assessed the expression profile of 18 HCV-related primary HCCs and their corresponding HCV-positive non-HCC counterpart, 1 HCV-positive liver sample without the corresponding HCC tissue, 14 gastrointestinal liver metastases and their corresponding non-cancerous tissue and 6 liver biopsies from patients with benign pathologies and normal liver by use of high-density oligonucleotide arrays. This represents an independent study from a previous study performed by our group [17]. An HCC-specific molecular signature set was identified that may enhance conventional pathologic assessment and may provide a tool for prognostic purposes, as well as identify targets for new therapeutic strategies.

Materials and Methods

Patient and Tissue Samples

A total of 102 liver human samples have been analyzed. Thirty one samples were used to define the signature genes in the first group of samples represented by a subset of samples from 19 patients profiled and reported in a previous study of molecular classification of HCV-related hepatocellular carcinoma [17]. An independent set of 71 liver biopsies has been used to define/evaluate the identified liver cancer signature (Figure 1).

Liver biopsies from 19 HCV-positive HCCs, 14 metastases from distant primary and 6 HCV-negative control samples from healthy donors obtained during laparoscopic cholecystectomy were obtained with informed consent at the liver unit of the INT "Pascale", Naples. In addition from each of the HCV-positive HCC and metastatic patients a paired liver biopsy from non-adjacent, non-tumor containing liver was obtained. All liver biopsies were stored in RNA Later at $-80^\circ$C (Ambion, Austin, TX). Confirmation of the histopathological nature of the biopsies was performed by the Pathology lab at INT before processing samples for RNA extraction. The non-HCC tissues from HCV-positive patient represented a heterogeneous sample consistent with the prevalent liver condition of each subject (ranging from persistent HCV-infection to cirrhotic lesions). One HCC sample, was shown to be mainly cirrhotic tissue and removed from the analysis. Furthermore, laboratory analysis confirmed that the 6 controls were seronegative for HCV antibodies.

Figure 1. Study design. Gene signature distinguishing the different pathological stage of liver disease and potential molecular progression markers was defined in the first dataset (left panel) and defined/evaluated in the independent second dataset (right panel). doi:10.1371/journal.pone.0056153.g001
Figure 2. Unsupervised hierarchical clustering: Panel A. Heat map based on 8,210 genes of 71 liver samples (HCV-related HCC, non-HCC counterpart, metastases, non cancerous counterpart and controls from healthy donors). Genes were filtered according to the following criteria: presence in 80% of all experiments, a ≥3 fold change ratio in at least one experiment. Red indicates over-expression; green indicates under-expression; black indicates unchanged expression; gray indicates no detection of expression (intensity of both Cy3 and Cy5 below the cutoff value). Each row represents a single gene; each column represents a single sample. The dendrogram at the top of the matrix indicates the degree of similarity between samples. Panel B Principal Component Analysis (PCA) 3D view for gene expression profiles from 71 liver samples. The PCA is based on log2 ratios and the expression profiles are across all the 36,000 genes in the microarrays. The green, blue, red, purple, and orange dots indicate metastasis, HCV-related HCC, HCV related non HCC, non metastatic counterpart, HCV-negative normal control and samples, respectively. doi:10.1371/journal.pone.0056153.g002

Figure 3. Heat map of the genes differentially expressed: identified by Class Comparison Analysis. Analysis including HCV-related HCC and normal liver samples from control subjects (Panel A); analysis including HCV-related non-HCC liver tissues and liver samples from control subjects (Panel B); analysis including liver metastasis and liver samples from control subjects (Panel C); analysis including HCV-related HCC and their HCV-positive/cirrhotic counterpart (Panel D); analysis including all HCV-related HCC, their HCV-positive/cirrhotic counterpart and normal liver samples, each column represents the average of all samples belonging to the same group as it was a single array (Panel E). The expression pattern of the genes is shown, each row representing a single gene. doi:10.1371/journal.pone.0056153.g003
### Table 1. Gene Set Characterizing HCV related HCC Samples.

| UGCluster | Gene Name | Description | First Dataset | Second Dataset |
|-----------|-----------|-------------|---------------|---------------|
|           | ARHGAP9  | Rho GTPase activating protein 9 | 0.000778 | 0.000521 |
|           | BIRC3     | Baculoviral IAP repeat-containing 3 | 0.007263 | 0.000341 |
|           | BLVRA     | Biliverdin reductase A | 0.001564 | 0.006145 |
|           | CCDC34    | Coiled-coil domain containing 34 | 0.009417 | 0.003457 |
|           | CK8       | Creatine kinase, brain | 0.00582 | 0.000255 |
|           | GPC3      | Glypican 3 | 0.005205 | 0.006609 |
|           | H2AFV     | H2A histone family, member V | 2.50E-05 | 0.000491 |
|           | HLA-F     | Major histocompatibility complex, class I, F | 0.003041 | 0.001327 |
|           | IFI6      | Interferon, gamma-inducible protein 16 | 0.008685 | 2.48E-08 |
|           | IFI27     | Interferon, alpha-inducible protein 27 | 7.06E-05 | 0.000336 |
|           | ISG15     | ISG15 ubiquitin-like modifier | 0.004877 | 0.005316 |
|           | JRK       | Jerky homolog | 0.002148 | 1.21E-05 |
|           | PLVAP     | Plasmalemma vesicle associated protein | 0.009883 | 0.004255 |
|           | PSIP1     | PC4 and SFRS1 interacting protein 1 | 0.000938 | 1.59E-06 |
|           | RELB      | V-rel reticuloendotheliosis viral oncogene homolog B | 0.002434 | 0.000175 |
|           | SERPIN1   | Serpin peptidase inhibitor | 0.002656 | 0.000266 |
|           | SMARCA4   | SWI/SNF related | 0.006768 | 0.002634 |
|           | STAT1     | Signal transducer and activator of transcription 1, 91 kDa | 1.58E-06 | 0.000374 |
|           | TAP1      | Transporter 1, ATP-binding cassette | 0.002018 | 0.000257 |
|           | THY1      | Thy-1 cell surface antigen | 0.00094 | 0.005523 |
|           | TK1       | Thymidine kinase 1 | 0.002458 | 0.000584 |
|           | UBD       | Ubiquitin D | 0.002593 | 0.000516 |

### Table 2. Gene Set Characterizing HCV related non HCC Samples.

| UGCluster | Gene Name | Description | First Dataset | Second Dataset |
|-----------|-----------|-------------|---------------|---------------|
|           | ARL8A     | ADP-ribosylation factor-like 8A | 0.001616 | 0.006094 |
|           | HCG4      | HLA complex group 4 | 3.64E-05 | 7.40E-06 |
|           | HERC6     | Hect domain and RLD 6 | 5.20E-05 | 0.001327 |
|           | HLA-DQA2  | Major histocompatibility complex, class II, DQ alpha 2 | 0.006389 | 0.005283 |
|           | HLA-F     | Major histocompatibility complex, class I, F | 4.84E-05 | 0.000372 |
|           | HLA-G     | Major histocompatibility complex, class I, G | 0.001323 | 0.001857 |
|           | IFI6      | Interferon, alpha-inducible protein 6 | 0.000191 | 0.006532 |
|           | ISG15     | ISG15 ubiquitin-like modifier | 0.000179 | 0.000515 |
|           | KIAA0746  | KIAA0746 protein | 0.001081 | 0.002704 |
|           | MX1       | Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 | 0.001325 | 3.40E-06 |
|           | OAS1      | 2',5'-oligoadenylate synthetase 1, 40/46 kDa | 0.000247 | 0.000107 |
|           | OAS1      | 2',5'-oligoadenylate synthetase-like | 0.00174 | 0.000355 |
|           | OASL      | 2',5'-oligoadenylate synthetase-like | 0.000174 | 3.40E-06 |
|           | OAS1      | 2',5'-oligoadenylate synthetase-like | 0.000247 | 0.000107 |
|           | STAT1     | Signal transducer and activator of transcription 1, 91 kDa | 7.86E-09 | 0.000204 |
|           | TAP1      | Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) | 0.000245 | 9.60E-05 |
|           | UBD       | Ubiquitin D | 0.002371 | 0.002236 |
|           | USP18     | Ubiquitin specific peptidase 18 | 0.008324 | 0.006233 |
RNA Isolation, cDNA Synthesis, in vitro Transcription and aRNA Labeling

Samples were homogenized in disposable tissue grinders (Kendall, Precision). Total RNA was extracted by TRIzol solution (Life Technologies, Rockville, MD), and purity of the RNA preparation was verified evaluating the 260:280 nm ratio of the spectrophotometric reading with NanoDrop (Thermo Fisher Scientific, Waltham, MA). Moreover, the integrity of extracted RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), analyzing the presence of 20S and 18S ribosomal RNA bands and verifying that the 20S/18S rRNA intensity ratio was equal or close to 1.5. In addition, phenol contamination was evaluated considering acceptable a 260:230 nm OD ratio within a 2.0–2.2 range.

Double-stranded complementary DNA (cDNA) was prepared from 3 μg of total RNA (T-RNA) in 9 μl DEPC-treated H2O using the Super script II Kit (Invitrogen) with a T7-(dT15) oligonucleotide primer. cDNA synthesis was completed at 42°C using the Super script II Kit (Invitrogen) with a T7-(dT15) primer and the oligo-dT-T7 primer and the resulting dsDNA was synthesized incubating the produced cDNA with 2 U of RNase-H (Promega) and 3 μl of Advantage cDNA Polymerase Mix (Clontech), in Advantage PCR buffer (Clontech), in presence of 10 mM dNTP and DNase-free Advantage cDNA Polymerase Mix (Clontech). The two labeled aRNA probes were separated from unincorporated nucleotides by filtration, fragmented, mixed and precipitated with ethanol in the presence of 1 μg of total RNA (T-RNA) in 9 μl DEPC-treated H2O and aRNA (amplified-RNA) was synthesized using Ambion’s T7 MegaScript in Vitro Transcription Kit (Ambion, Austin, TX). aRNA recovery and removal of template dsDNA was achieved by TRIzol purification. For the second round of amplification, aliquots of 1 μg of the aRNA were reverse transcribed into cDNA using 1 μl of random hexamer under the conditions used in the first round. Second-stand cDNA synthesis was initiated by 1 μg oligo-dT-T7 primer and the resulting dsDNA was used as template for in vitro transcription of aRNA in the same experimental conditions as for the first round [18]. 6 μg of this aRNA was used for probe preparation, in particular test samples were labeled with USL-Cy5 (Kreatech) and pooled with the same amount of reference sample (control donor peripheral blood mononuclear cells, PBMC, seronegative for anti-hepatitis C virus (HCV) antibodies ) labeled with USL-Cy3 (Kreatech). The two labeled aRNA probes were separated from unincorporated nucleotides by filtration, fragmented, mixed and co-hybridized to a custom-made 36K oligoarrays at 42°C for 24 h. The oligo-chips were printed at the Immunogenetics Section, National Institutes of Health (Bethesda, MD). After hybridization the slides were washed with 2xSSC/0.1%SDS for 1 min, 1xSSC for 1 min, 0.2xSSC for 1 min, 0.05xSSC for 10 sec., and dried by centrifugation at 800 g for 3 minutes at RT.

Data Analysis

Hybridized arrays were scanned at 10-μm resolution with a GenePix 4000 scanner (Axon Instruments) at variable photomultiplier tube (PMT) voltage to obtain maximal signal intensities with less than 1% probe saturation. Image and data files were deposited at microarray data base (mAdb) at http://madb.nci.nih.gov and retrieved after median centered, filtering of intensity (>200) and spot elimination. Data were further analyzed using Cluster and TreeView software (Stanford University, Stanford, CA).

Statistical Analysis

Unsupervised Analysis. For this analysis, a low-stringency filtering was applied, selecting the genes differentially expressed in 80% of all experiments with a >3 fold change ratio in at least one experiment. 8,210 genes were selected for the analysis including the five groups of analyzed samples the HCV-related HCC, their non-HCC counterpart, metastasis, and their non metastatic counterpart as well as samples from the normal controls. Hierarchical cluster analysis was conducted on these genes according to Eisen et al. [19], differential expressed genes were visualized by Treeview and displayed according to the central method [20]. Principal component analysis (PCA) was applied for visualization when relevant based on the complete dataset.

Supervised Analysis. Supervised class comparison was performed using the BRB ArrayTool developed at NCI, Biometric Research Branch, Division of Cancer Treatment and Diagnosis. Three subsets of genes were explored. The first subset included genes up-regulated in HCV-related HCC compared to normal control samples, the second subset included genes up-regulated in the HCV-related non-HCC counterpart compared with normal control samples, the third subset included genes up-regulated in metastasis compared with normal control samples. Paired samples were analyzed using a two-tailed paired Student’s t-Test. Unpaired samples were tested with a two-tailed unpaired Student’s t-Test assuming unequal variance or with an F-test as appropriate. All analyses were tested for an univariate significance threshold set at a p-value <0.01. Gene clusters identified by the univariate t-test were challenged with two alternative additional tests, a univariate permutation test (PT) and a global multivariate PT. The multivariate PT was calibrated to restrict the false discovery rate to 10%. Genes identified by univariate t-test as differentially expressed (p-value <0.01) and a PT significance <0.05, were considered truly differentially expressed. Gene function was
| UGCluster | Gene Name | Description | p-value | Fold-Change |
|-----------|-----------|-------------|---------|-------------|
| Hs.529353 | ACSS1     | Acyl-CoA synthetase short-chain family member 1 | 3.99E-08 | 4.40 |
| Hs.514581 | ACTG1     | Actin, gamma 1 | 2.01E-08 | 3.32 |
| Hs.530009 | AGR2      | Anterior gradient homolog 2 | 0.00026377 | 3.89 |
| Hs.270833 | AREG      | Amphiregulin | 3.82E-05 | 3.82 |
| Hs.701324 | ARHGAP4   | Rho GTPase activating protein 4 | 1.01E-07 | 2.59 |
| Hs.371889 | ATP1A1    | ATPase, Na+/K+ transporting, alpha 1 polypeptide | 6.78E-08 | 2.98 |
| Hs.919382 | BSG       | Basigin | 1.05E-07 | 2.93 |
| Hs.8859  | CANT1     | Calcium activated nucleotidease 1 | 8.44E-06 | 2.90 |
| Hs.523852 | CCND1     | Cyclin D1 | 7.16E-06 | 3.28 |
| Hs.82916 | CCT6A     | Chaperon containing TCP1, subunit 6A (zeta 1) | 1.06E-08 | 4.3 |
| Hs.502328 | CD44      | CD44 molecule | 0.00033064 | 2.16 |
| Hs.114286 | CD9       | CD9 molecule | 4.99E-07 | 3.53 |
| Hs.709226 | CDH3      | Cadherin 3, type 1, P-cadherin | 2.32E-07 | 3.37 |
| Hs.712603 | CENPO     | Centromere protein 0 | 4.28E-09 | 2.92 |
| Hs.173724 | CKB       | Creatine kinase, brain | 7.68E-09 | 5.14 |
| Hs.414565 | CLC1      | Chloride intracellular channel 1 | 4.93E-07 | 3.23 |
| Hs.443625 | COL3A1    | Collagen, type III, alpha 1 | 8.67E-07 | 6.38 |
| Hs.17441  | COL4A1    | Collagen, type IV, alpha 1 | 1.20E-06 | 5.41 |
| Hs.570065 | COL4A3    | Collagen, type IV, alpha 3 (Goodpasture antigen) | 0.00010556 | 3.13 |
| Hs.2242  | CSN2      | Casein beta | 1.69E-05 | 3.20 |
| Hs.67928 | ELF3      | E74-like factor 3 | 1.60E-05 | 3.01 |
| Hs.655278 | FER1L3    | Fer-1-like 3, myoferlin | 4.01E-08 | 2.86 |
| Hs.443687 | FHL2      | Four and a half LM domains 2 | 1.35E-05 | 3.20 |
| Hs.499659 | GARNL4    | GTPase activating Rap/RanGAP domain-like 4 | 2.00E-07 | 4.21 |
| Hs.203699 | GOLPH3L   | Golgi phosphoprotein 3-like | 6.84E-09 | 12.95 |
| Hs.231320 | GPR160    | G protein-coupled receptor 160 | 4.64E-08 | 3.70 |
| Hs.2704  | GPX2      | Glutathione peroxidase 2 | 4.41E-06 | 2.81 |
| Hs.523836 | GSTP1     | Glutathione-S-transferase pi | 2.26E-07 | 6.93 |
| Hs.31720 | HEPH      | Hephaestin | 1.35E-05 | 3.96 |
| Hs.594238 | KPN2A     | Karyopherin alpha 2 (RAG cohort 1, importin alpha 1) | 0.00037649 | 2.15 |
| Hs.406013 | KRT18     | Keratin 18 | 1.74E-06 | 5.81 |
| Hs.374191 | LEPREL1   | Leprecan-like 1 | 3.36E-05 | 10.35 |
| Hs.5302  | LGALS4    | Lectin, galactoside-binding, soluble, 4 (galectin 4) | 2.74E-05 | 2.59 |
| Hs.444118 | MCM6      | Minichromosome maintenance complex component 6 | 7.33E-09 | 2.66 |
| Hs.632177 | MVP       | Major vault protein | 6.08E-07 | 2.59 |
| Hs.533282 | NONO      | Non-POU domain containing, octamer-binding | 4.45E-08 | 2.55 |
| Hs.406515 | NQO1      | NAD(P)H dehydrogenase, quinone 1 | 0.00017945 | 9.82 |
| Hs.553765 | OR4K17    | Olfactory receptor, family 4, subfamily K, member 17 | 3.42E-05 | 3.57 |
| Hs.154104 | PLAGL2    | Pleiomorphic adenoma gene-like 2 | 2.41E-08 | 2.50 |
| Hs.155342 | PRKCD     | Protein kinase C, delta | 4.30E-08 | 2.52 |
| Hs.499094 | PYCARD    | PYD and CARD domain containing | 6.41E-07 | 2.52 |
| Hs.714383 | RAPGEFL1  | Rap guanine nucleotide exchange factor (GEF)-like 1 | 0.00091543 | 2.67 |
| Hs.449968 | RHCE      | Rh blood group, CcEe antigens | 0.00022816 | 3.64 |
| Hs.653288 | RNF12     | Ring finger protein 12 | 7.53E-09 | 2.78 |
| Hs.381061 | RPL19     | Ribosomal protein L19 | 2.49E-08 | 2.77 |
| Hs.514196 | RPL27     | Ribosomal protein L27 | 1.36E-08 | 2.63 |
| Hs.178551 | RPL8      | Ribosomal protein L8 | 3.73E-07 | 2.56 |
| Hs.356502 | RPLP1     | Ribosomal protein, large, P1 | 2.73E-07 | 2.88 |
| Hs.118076 | RPS4X     | Ribosomal protein S4, X-linked | 1.03E-08 | 2.51 |
Time course analysis. A time course analysis was performed to identify markers of tumoral progression between normal liver samples, HCV-related non HCC and HCV-related HCC liver samples. For this analysis, normal liver samples (CTR) were taken as the early time point, HCV-related non HCC as the intermediate point and the HCV-related HCC as the last time point. Parameters for gene selection were: F test \( p \)-value <0.005, 80% presence call, ratio of >2 and false discovery rate <0.1.

Ingenuity pathway analysis (IPA). Pathway analysis was performed using the gene set expression comparison kit implemented in BRB-Array-Tools. The human pathway lists determined by “Ingenuity System Database” was selected. Significance threshold of t-test was set at 0.01. IPA is a system that transforms large dataset into a group of relevant networks containing direct and indirect relationships between genes based on known interactions in the literature. The significance of each network was estimated by the scoring system provided by Ingenuity. The scores are determined by the number of differentially expressed genes within each of the networks and the strength of the associations among network members.

Results

Genes Differentially Expressed among Distinct Tissues

The gene expression profile- of tissue samples from the various groups (HCV-related HCC, non-HCC counterpart, metastases, non-metastatic counterpart and controls from healthy donors) were compared by unsupervised analysis. Genes were filtered according to the following criteria: presence in 80% of all experiments, a \( > 3 \) fold change ratio in at least one experiment; this filter yielded 8,210 genes that were used for clustering. The HCC and the metastatic samples prevalently clustered into distinct groups, based on differences in their patterns of gene expression (Figure 2A). PCA segregated the different sample types into four-five groups according on their pathological status. Statistical and functional analysis of the profiles identified a set of genes whose expression was differentially altered between the groups (Figure 2B). The expression pattern of gastrointestinal liver metastases was clearly distinct from that of HCV-related primary HCC, allowing a definite molecular characterization of the two diseases.

Differential Gene Expression Patterns between HCV positive Liver Tissue with and without HCC, Metastasis and Normal control Liver Tissue

An unpaired Student’s t-test with a cut-off \( p \)-value set at \( p < 0.01 \) comparing HCV-related HCC to normal controls indentified 1864 genes differentially expressed. Among them, 993 were up-regulated and 871 down-regulated in HCV-related HCCs (Figure 3A).

In total 198 genes showing up regulation were found in common with our previous study [17], the results is presented as two-way Venn diagram in additional Figure S1A and Supplemental Table S1. The common genes 2.0 fold upregulated (ranked according to the name) are listed in Table 1. Comparison between liver tissues from HCV-related non HCC and normal controls (\( p < 0.01 \)) identifies 1526 genes differentially expressed. Among them, 618 were up-regulated and 918 down-regulated in HCV-related liver tissues (Figure 3B). In total 59 genes showing up regulation were found in common with our previous study [17], the results is presented as two-way Venn diagram in additional Figure S1B and Supplemental Table S1. The common genes 2.0 fold upregulated (ranked according to the name) are listed in Table 2.
Table 5. Gene-signature of HCC progression.

| UGCluster | Name                                                                 | Symbol     |
|-----------|----------------------------------------------------------------------|------------|
| Hs.654433 | Alcohol dehydrogenase 1A (class I), alpha polypeptide               | ADH1A      |
| Hs.477887 | Angiotensin II receptor, type 1                                      | AGTR1      |
| Hs.116724 | Aldo-keto reductase family 1, member B10                            | AKR1B10    |
| Hs.9613   | Angiopoietin-like 4                                                 | ANGPTL4    |
| Hs.374774 | Ankyrin repeat domain 29                                            | ANKRD29    |
| Hs.633003 | Apolipoprotein A-I                                                 | APOA1      |
| Hs.440934 | Arginase, liver                                                     | ARG1       |
| Hs.486031 | Activating signal cointegrator 1 complex subunit 3                 | ASCC3      |
| Hs.98338  | Aurora kinase C                                                    | AURKC      |
| Hs.80756  | Betaine-homocysteine methyltransferase                              | BHMT       |
| Hs.356440 | Coiled-coil domain containing 72                                   | CCDC72     |
| Hs.575869 | Complement factor H-related 1                                      | CFHR1      |
| Hs.591464 | Cingulin                                                            | CGN        |
| Hs.268326 | C-type lectin domain family 2, member D                             | CLEC2D     |
| Hs.220649 | C-type lectin superfamily 4, member G                               | CLEC4G     |
| Hs.172928 | Collagen, type I, alpha 1                                           | COL1A1     |
| Hs.508716 | Collagen, type IV, alpha 2                                          | COL4A2     |
| Hs.522891 | Chemokine (C-X-C motif) ligand 12                                   | CXCL12     |
| Hs.590921 | Chemokine (C-X-C motif) ligand 2                                    | CXCL2      |
| Hs.706262 | Decorin                                                             | DCN        |
| Hs.476453 | Deoxyribonuclease I-like 3                                          | DNASE1L3   |
| Hs.80552  | Dermatopontin                                                      | DPT        |
| Hs.224171 | Enolase 3 (beta, muscle)                                            | ENO3       |
| Hs.27836  | Fibronectin type III domain containing 4                            | FNDC4      |
| Hs.25647  | V-fos FBX murine osteosarcoma viral oncogene homolog                | FOS        |
| Hs.78619  | Gamma-glutamyl hydrolase                                           | GGH        |
| Hs.518525 | Glutamate-ammonia ligase                                           | GLUL       |
| Hs.713537 | Glypican 3                                                         | GPC3       |
| Hs.190783 | Histidine ammonia-lyase                                             | HAL        |
| Hs.190783 | Histidine ammonia-lyase                                             | HAL        |
| Hs.659767 | Hydroxyacid oxidase 2                                               | HAO2       |
| Hs.59889  | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2                    | HMGCS2     |
| Hs.532634 | Interferon, alpha-inducible protein 27                             | IFI27      |
| Hs.523414 | Insulin-like growth factor 2 (somatomedin A)                        | IGF2       |
| Hs.709180 | Netrin 2-like (chicken)                                             | IGRC       |
| Hs.632629 | Indolethylamine N-methyltransferase                                 | INMT       |
| Hs.657894 | IQ motif containing H                                               | IQCH       |
| Hs.458485 | ISG15 ubiquitin-like modifier                                       | ISG15      |
| Hs.76716  | Inter-alpha (globulin) inhibitor H3                                 | ITIH3      |
| Hs.77741  | Kininogen 1                                                        | KNG1       |
| Hs.77741  | Kininogen 1                                                        | KNG1       |
| Hs.492314 | Lysosomal associated protein transmembrane 4 beta                   | LAPTMB4    |
| Hs.130767 | Leucine rich repeat containing 46                                   | LRRC46     |
| Hs.482087 | Leucine rich repeat containing 8 family, member D                  | LRRCD8     |
| Hs.514402 | Protein kinase LYK5                                                | LYK5       |
| Hs.201083 | Mal, T-cell differentiation protein 2                               | MAL2       |
| Hs.349110 | Macrophage stimulating 1 (hepatocyte growth factor-like)            | MST1       |
| Hs.512587 | Macrophage stimulating 1 (hepatocyte growth factor-like)            | MST1       |
| Hs.512973 | Protein tyrosine phosphatase-like A domain containing 1             | PTPLAD1    |
Canonical Pathways and Molecular and Cellular Functions

To explore the biological significance of the genes characterizing different pathological or normal conditions we investigated their interactions by IPA mapping their molecular/cellular functions and canonical pathways. The more important molecular and cellular functions (ranked according to lowest p value) of genes up-regulated in HCV-related HCC samples were related to regulation of gene expression (1.12E-17 to 3.41E-03), cellular growth and proliferation (2.00E-14 to 3.84E-03) and post-translational modification (1.53E-09 to 2.45E-03). The top canonical pathways included protein ubiquitination (p = 2.88E-03), 14-3-3 mediated Signaling (p = 1.15E-02) and Aryl Hydrocarbon receptor signaling pathway (p = 3.09E-02) (Figure 4A). The more important molecular and cellular functions (High p value) of genes up-regulated in HCV-related non HCC samples were related to Cellular Growth and Proliferation (1.04E-22 to 4.61E-04), Gene Expression (2.07E-22 to 4.58E-04) and Cell-To-Cell Signaling and Interaction (9.05E-14 to 4.65E-04). The top canonical pathways included Interferon Signaling Genes (p = 9.78E-04), Antigen Presentation Pathway (p = 1.38E-03) and Protein Ubiquitination Pathway (p = 2.44E-02) (Figure 4B).

The more important molecular and cellular functions (High p value) of genes up-regulated in metastases were related to Gene Expression (6.08E-26 to 2.00E-04), Cellular Growth and Proliferation (1.86E-25 to 8.64E-05), Cell Cycle (5.67E-21 to 1.33E-04). The top canonical pathways included Arginine and Proline Metabolism (p = 1.77E-09), Coagulation System (p = 9.68E-09) and Acute Phase Response Signaling (p = 2.08E-08) (Figure 4C). Table 6 summarizes the more important findings for each of the described comparison analysis.

Among the three different class comparison analysis (HCV-related HCC, HCV-related non HCC and Metastatic liver tissue vs normal control) we found a gene-set that distinguish the different cases of liver disease, in particular with time course analysis we identify the genes that should be candidate as a possible progression markers (Figure 5).

Discussion

HCC is a common and aggressive malignant tumor worldwide with a dismal outcome. Early detection and resection may offer an opportunity to improve the long-term survival for HCC patients. Unfortunately, with current diagnostic approaches, only about 10% to 20% of HCC patients are eligible for resection [21]. In the first study, microarray analyses of liver biopsies from HCC nodules and paired non-adjacent non-HCC liver tissue of the same HCV-positive patients were compared to biopsies from HCV-negative control subjects. The class comparison analysis used in that study successfully identified a set of genes significantly differentially expressed. Moreover
the up-regulated genes identified within the individual class comparison analysis were evaluated and classified by a pathway analysis, according to the "Ingenuity System Database".

The genes up-regulated in samples from HCV-related HCC were classified in metabolic pathways, and the most represented are the Aryl Hydrocarbon receptor signaling (AHR) and, protein Ubiquitination pathways, which have been previously reported to be involved in cancer, and in particular in HCC, progression. The genes up-regulated in samples from HCV-related non-HCC tissue were classified in several pathways prevalently associated to inflammation and native/adaptive immunity and most of the over expressed genes belong to the Antigen Presentation pathway. In this new study we performed the same statistical analysis under the same condition to confirm our previous data. To elucidate the genes and molecular pathways involved in the HCV-related HCC a class comparisons analysis were performed on new samples set. This analysis allowed us to identify the unique probe sets characterizing the pathological status, in fact as expected, the gene expression patterns were found to vary significantly among the HCC and normal control liver samples. Genes associated with cell death, cell to cell signaling and interaction, were found to have increased expression in HCC samples. The molecular events linked to the development and progresses of HCC are not well known. Malignant hepatocytes are the result of sequential changes accumulated in mature hepatocytes or can derive from stem cells. The most accepted hypotheses [22,23] describes a step-by-step process in which external stimuli induce genetic alterations in mature hepatocytes leading to cell death, cellular proliferation, and the production of monoclonal populations. These populations harbor dysplastic hepatocytes that evolve to dysplastic nodules [24].

Canonical pathways prevalently associated with HCV-related HCC included protein ubiquitination, antigen presentation and Aryl Hydrocarbon receptor signaling pathway, confirming our previous data.

Figure 4. Ingenuity Pathways analysis: significant pathways at the nominal 0.01 level of the unpaired Student’s t-test. The 3 top-scoring pathways of genes up regulated in HCV-related HCC (Panel A), in HCV related non HCC (Panel B) and in Metastatic liver samples (Panel C).

doi:10.1371/journal.pone.0056153.g004
Cellular growth and proliferation and antigen presentation were the more important cellular and molecular functions when HCV-related non HCC samples were compared with normal control liver tissue. These data agree with the numerous regulatory roles reported for the HCV core, that affect signal transduction, expression of viral and cellular genes, cell growth and proliferation [25,26].

Several viruses target specific components of the MHC class I pathway, leading to diminished cell surface expression of MHC class I molecules. Other viruses block the transport of MHC class I molecules through the endoplasmic reticulum (ER), inhibit TAP-mediated translocation of cytoplasmic peptides into the ER, or interfere with proteasomal degradation of their own proteins [27]. Other viruses, like human cytomegalovirus, escape CD8+ T-cell recognition by downregulating cellular MHC class I molecules [28] and simultaneously inducing the expression of virus-encoded MHC class I homologues capable of engaging inhibitory receptors that give a negative signal blocking NK cell function. Flaviviruses can up regulate MHC class I cell surface expression by increased peptide supply to the ER [29,30]. Viruses may use these strategies to evade and counteract a potential NK cell attack. Some studies demonstrated that HCV core protein induced the up regulation of antigen presentation and immune response mechanisms [31].

Canonical pathways mainly associated with HCV-related non-HCC tissue included Interferon Signaling, SAPK/JNK Signaling and NF-κB Activation by viruses pathway. These pathways are prevalently associated with inflammation and native/adaptive immunity.

A traditional HCC diagnosis has relied on the use of a single biomarker approach (e.g., AFP).

Advances in gene expression profiling have provided new insights into the molecular genetics of HCC, showing strong expression signatures of cell proliferation and antiapoptosis genes (such as PDGFRα and BCL2) along with genes involving ubiquitination [32], as well as unique molecular markers of progression like HSP70, CAP2, GPC3, and GS [33]. HCC-specific alterations of signal transduction pathways and protein expression patterns have been detected and have led to the development of new therapeutic agents with molecular targets such as EGFR, VEGF, DDEFL, VANGL1, WDRPUH, Ephrin-A1, GPC3, and GS [33,34].

Among the four different class comparison analysis (HCV-related HCC, HCV-related non HCC and Metastatic liver tissue vs. normal control; HCV-related HCC vs. autologous HCV-related HCC), cellular growth and proliferation were the most important functions. The number of differentially expressed genes in each class comparison was statistically significant with a p-value of 1.04E -04 (9.05E -21 to 4.61E -04) for HCV-related HCC tissues vs. autologous HCV-related HCC, and for HCV-related non HCC samples vs. normal control liver tissue.

### Table 6.

| HCV related HCC tissues versus normal livers | HCV related non HCC tissues versus normal livers | Metastatic liver tissues versus normal livers |
|-------------------------------------------|-----------------------------------------------|---------------------------------------------|
| Number of differentially expressed genes  | Associated Network Functions (five top-scorer functions) | 1. Cellular Assembly and Organization, Genetic Disorder, Lipid Metabolism |
| Gene Expression (1.12E – 17 to 3.41E – 03) 1074 genes; | Cellular Growth and Proliferation (1.04E – 22 to 4.61E – 04) 1353 genes; | Gene Expression (6.08E – 26 to 2.00E – 04) 1299 genes; |
| Cellular Growth and Proliferation (2.00E – 14 to 3.84E – 03) 1328 genes; | Gene Expression (2.07E – 22 to 4.58E – 04) 1066 genes; | Cellular Growth and Proliferation (1.86E – 25 to 8.64E – 05) 1623 genes; |
| Post-Translational Modification (1.53E – 09 to 2.45E – 03) 523 genes; | Cell-To-Cell Signaling and Interaction (9.05E – 14 to 4.65E – 04) 681 genes; | Cell Cycle (5.67E – 21 to 1.33E – 04) 818 genes; |
| Cell Cycle (1.94E – 08 to 3.81E – 03) 624 genes; | Cell Death (3.94E – 13 to 4.59E – 04) 1032 genes; | Cellular Movement (6.06E – 21 to 2.17E – 04) 911 genes; |
| Cell Morphology (4.44E – 08 to 4.22E – 03) 393 genes; | Cellular Movement (1.43E – 12 to 4.80E – 04) 745 genes; | Cell Death (9.05E – 18 to 1.71E – 04) 1266 genes; |

### Top canonical pathways

| Pathway | Function |
|---------|----------|
| Protein Ubiquitination Pathway (p = 2.88E – 03); | | Arginine and Proline Metabolism (p = 1.77E – 09); |
| 14-3-3 mediated Signaling (p = 1.13E-02); | | Coagulation System (p = 9.68E – 09); |
| Aryl Hydrocarbon receptor signaling (p = 3.09E – 02); | | Acute Phase Response Signaling (p = 2.08E – 08); |
non HCC liver tissue) we found a gene-set that distinguish the different cases of liver disease, in particular with time course analysis we identify the genes that should be candidate as a possible progression markers (e.g., GPC3, CXCL12, SPINK1, GLUL, UBD, TM4SF5, DPT, SCD, MAL2, TRIM55, COL4A2). All these data altogether suggested developing a specific gene-chip along with genes showing the highest fold up-regulation in common with previous work representing the different stage of disease.

The identification of the lesions and the evaluation of their neoplastic progression will be based on the gene pattern expression on the gene-chip (Figure 5, Table 1–5).

In conclusion we identified a set of genes highly candidate as gene signatures to be validate on a larger clinical sample size of liver tissue biopsies to evaluate consistency and universality of the results, to verify the effective power of distinguishing different pathological stage of liver disease and to assess their value as progression markers for early HCC diagnosis in HCV positive patients. Furthermore, identification of specific alterations in key metabolic pathways could give the opportunity to identify new therapeutic targets for innovative, personalized treatments.

Moreover, the gene-expression pattern will be correlated with additional clinical parameters (besides disease stage and tumor histopathology) such as the frequency with which patients show the identified profile, patient age and gender, concurrent diseases and pharmacological treatments.

In parallel, all our liver collection samples will undergo further molecular analysis (which include miRNA, aCGH, proteomic) to develop increasingly sophisticated gene expression indicators of specific types or stages of liver disease as well as responsibility to pharmacological treatment of HCV-associated hepatitis and their progression to cirrhosis/HCC.

**Supporting Information**

**Figure S1** Venn diagram illustrating the number of up-regulated genes in common between first (green circle) and second (blue circle) data set. Genes in common are in red circle. A) Comparison analysis between HCV-related HCC versus normal liver. B) Comparison analysis between HCV related non-HCC versus normal liver. C) Comparison analysis between HCV-related HCC versus autologous HCV related non-HCC.

**Table S1** List of the 198 genes upregulated in HCC samples of the two independent datasets, in comparison to liver control biopsies.
Table S2  List of genes upregulated or down regulated in metastatic liver lesions in comparison to normal liver tissue.

Author Contributions
Conceived and designed the experiments: FMB FI FMM. Performed the experiments: VDG MT EW. Analyzed the data: VDG EW AW FMB. Contributed reagents/materials/analysis tools: MT FMB EW FMM. Wrote the paper: VDG EW FMB.

References
1. El-Serag HB, Mason AC (1999) Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 340: 745–750.
2. Davila JA, Petersena NJ, Nelson HA, El-Serag HB (2003) Geographic variation within the United States in the incidence of hepatocellular carcinoma. J Clin Epidemiol 56: 487–493.
3. El-Serag HB (2002) Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 36: S74–S78.
4. Romeo R, Colombos M (2002) The natural history of hepatocellular carcinoma. Toxicology 181–182: 39–42.
5. Schafer DF, Sorrell MF (1999) Hepatocellular carcinoma. Lancet 353: 1253–1257.
6. Kim JW, Wang XW (2003) Gene expression profiling of preneoplastic liver and liver cancer: a new era for improved early detection and treatment of these deadly diseases? Carcinogenesis 24: 363–369.
7. Block TM, Mehta AS, Fimmel CJ, Jordan R (2003) Molecular viral oncology of hepatocellular carcinoma. Oncogene 22: 5093–5107.
8. Buendia MA (1998) Hepatitis B viruses and cancerogenesis. Biomed Pharmacother 52: 34–43.
9. Koike K, Moriya K, Kimura S (2002) Role of hepatitis C virus in the development of hepatocellular carcinoma: transgenic approach to viral hepatocarcinogenesis. J Gastroenterol Hepatol 17: 394–400.
10. Ohata K, Terasima S (2007) Synthesis and biological activity of enantiomeric pairs of 5-oxoiodoacetylcongeners. Bioorg Med Chem Lett 17: 4070–4074.
11. Brunt EM, Neuschwander-Tetri BA, Oliver D, Wehmeier KR, Bacon BR (2004) Nonalcoholic steatohepatitis: histologic features and clinical correlations with 30 blinded biopsy specimens. Hum Pathol 35: 1070–1082.
12. Davila JA, Morgan RO, Shah Y, McGlynn KA, El-Serag HB (2005) Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut 54: 533–539.
13. Al-Sukhun S, Hussain M (2003) Current understanding of the biology of advanced bladder cancer. Cancer 97: 2064–2075.
14. Theocharides D (2003) Molecular pathogenesis of urothelial bladder cancer. Histol Histopathol 18: 259–274.
15. Groen KA (1999) Primary and metastatic liver cancer. Semin Oncol Nurs 15: 40–57.
16. Yamané B, Weber S (2009) Liver-directed treatment modalities for primary and secondary hepatic tumors. Surg Clin North Am 89: 97–113, ix.
17. De Giorgi V, Monaco A, Worchech A, Torrielli M, Lizzo F, et al. (2009) Gene profiling, biomarkers and pathways characterizing HCV-related hepatocellular carcinoma. J Transl Med 7: 85.
18. Wang E, Miller LD, Ohmacht GA, Liu ET, Marincola FM (2000) High-fidelity mRNA amplification for gene profiling. Nat Biotechnol 18: 457–459.
19. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95: 14863–14868.
20. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, et al. (2000) Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet 24: 227–235.
21. Bai HC, Fan ST, Lo CM, Chu KM, Liu CL, et al. (1995) Hepatic resection for secondary hepatic tumors. Surg Clin North Am 89: 97–113, ix.
22. Hoshita Y, Villanueva A, Kobayashi M, Pric J, Chiang DY, et al. (2008) Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 359: 1995–2004.
23. Sakamoto M, Mori T, Masugi Y, Effendi K, Rie I, et al. (2008) Candidate molecular markers for histological diagnosis of early hepatocellular carcinoma. Intervirology 51 Suppl 1: 42–45.
24. Lohman V, Korner F, Koch J, Herian U, Thielmann L, et al. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285: 110–113.
25. Blight KJ, Kobylkalo AV, Reed KE, Agapov EV, Rice CM (1998) Molecular virology of hepatitis C virus: an update with respect to potential antiviral targets. Antivir Ther 3: 71–81.
26. Rosenberg W (1999) Mechanisms of immune escape in viral hepatitis. Gut 44: 759–764.
27. Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, et al. (2002) NK cell activity during human cytomegalovirus infection is dominated by UN2-11-mediated HLA class I down-regulation. J Immunol 169: 3257–3266.
28. Momburg F, Mullbacher A, Lobigs M (2001) Modulation of transporter associated with antigen processing (TAP)-mediated peptide import into the endoplasmic reticulum by flavivirus infection. J Virol 75: 5663–5671.
29. Mullbacher A, Lobigs M (1995) Up-regulation of MHC class I by flavivirus-induced peptide translocation into the endoplasmic reticulum. Immunity 3: 207–214.
30. Herzer K, Falk CS, Encke J, Eichhorn ST, Ulenheimer A, et al. (2003) Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p33 and TAP1 impacts natural killer cell cytotoxicity. J Virol 77: 8299–8309.
31. Yasugi Y, Sugiyama Y, Ahtotani H (2010) Molecular targets for liver cancer therapy: From screening of target genes to clinical trials. Hepatol Res 40: 49–60.
32. Okabe H, Furukawa Y, Kato T, Hasagawa S, Yamaoka Y, et al. (2004) Isolation of development and differentiation enhancing factor-like-1 (DDEF1) as a drug target for hepatocellular carcinomas. Int J Oncol 24: 43–48.
33. Okabe H, Satoh S, Furukawa Y, Kato T, Hasagawa S, et al. (2005) Involvement of PEG10 in human hepatocellular carcinogenesis through interaction with SIAH1. Cancer Res 65: 3043–3048.
34. Yagyu R, Hamamoto R, Furukawa Y, Okabe H, Yamamura T, et al. (2002) Isolation and characterization of a novel human gene, VANGL1, as a therapeutic target for hepatocellular carcinoma. Int J Oncol 20: 1173–1178.
35. Silva FP, Hamamoto R, Nakamura Y, Furukawa Y (2005) WDRPUH, a novel WD-repeat-containing protein, is highly expressed in human hepatocellular carcinoma and involved in cell proliferation. Neoplasia 7: 348–355.
36. Nakamukai Y, Ishikawa S, Iwamata H, Imanura T, Sakamoto H, et al. (2003) Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int J Cancer 103: 453–465.
37. Ito WK, Bai HC, Whong NL, To KF, et al. (2006) Novel identification of axin upregulations in the motile phenotype of hepatocellular carcinoma. Mod Pathol 19: 1108–1116.
38. Zender L, Kubicka S (2008) Molecular pathogenesis and targeted therapy of hepatocellular carcinoma. Oncology 31: 550–555.
39. Saka K, Yagi Y, Sugiyama Y, Ahtotani H (2010) Molecular targets for liver cancer therapy: From screening of target genes to clinical trials. Hepatol Res 40: 49–60.