Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Screening of HRas, RRas and FOS as Potential Pharmacodynamic Candidates for the Treatment of Hepatocellular Carcinoma

Savita Mishra
Amity University Amity Institute of Biotechnology
Sandhya Hora
National Institute of Biologicals
Deepshikha Pande Katare (dpkatare@amity.edu)
Amity University Amity Institute of Biotechnology https://orcid.org/0000-0002-1908-2072

Research Article

Keywords: Hepatocellular Carcinoma, HRas, FOS, RRas, hub gene, Protein-Protein interaction network, enrichment analysis, drug-gene interaction

Posted Date: March 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1453092/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Hepatocellular carcinoma (HCC) is a deadly primary tumour that begins in hepatocytes. Tumour cell proliferation and progression is influenced by cellular components of the liver and their microenvironment. Pharmacodynamic (PD) biomarkers are biomolecules that demonstrate the effect of a therapeutic intervention on the affected organ and its microenvironment. PD biomarkers are used to study drug target interactions and their biological responses. High-throughput technologies and bioinformatics tools are critical for interpreting proteomics data and exploring new biological insights into cellular processes, disease pathogenesis, and biomarker discovery. We have developed an animal model of HCC using DEN with 2AAF as a carcinogen and treated it with multiple doses of sorafenib-silibinin combinational tablets. With proteomic studies, we have screened differently expressed protein signatures as pharmacodynamic biomarkers. The PPI network was created on the STRING platform and visualised on Cytoscape software. The MCODE plug-in was implied to identify the modules with the highest significant score. Later, the CytoHubba plug-in was used to filter the top ten hub genes in each module. Finally Drug-gene interaction was done using hub gene on the STITCH platform for molecular analysis of combinational treatments. We have identified a total of 11 differentially expressed proteins with MALDI-TOF-MS. Bioinformatics analysis of these biological factors is used to identify genes that are helpful in treatment. The PPI network complex resulted in 141 nodes with 1520 edges. The top ten hub gene list revealed the significance of three query proteins (HRas, RRas, and FOS) in the protein network, which was later validated with overall survival analysis using the GEPIA tool. Finally, drug–gene interaction predicted that combinational treatment has a significant effect at the molecular level and, out of 11 differentially expressed proteins, 3 could be potential pharmacodynamic biomarker. Based on computational and proteomic analysis, we have screened the expression and therapeutic value of potential pharmacodynamic biomarkers (HRas, RRas, and FOS) with sorafenib-silibinin formulation treatment in the HCC model.

Introduction

The oncotherapeutics field has been transformed extensively after better understating of proteomics in cancer biology [1]. Pharmacodynamic biomarkers (PD) are direct indicator of drugs efficacy, target effect, and biological response of targeted therapy against tumour. Focused PD biomarker measurement provides critical information to select combinational molecular targeted agents as well as optimization of doses for the combinational regimen [2]. Hepatocellular carcinoma (HCC) is an aggressive primary tumor initiates from well differentiated hepatocytes. HCC ranked as the 2nd deadliest cancer and the 6th most frequent cancer due to poor prognosis and accounts almost 13% of all deaths worldwide [3]. The molecular targeted drug sorafenib has notably increased survival rate of HCC patients, but this drug has severe side effects. The major challenges with sorafenib monotherapy treatments are insufficient inhibition of mortality rate in HCC patients. Hence, there is a need of hour to discover an alternative approach for the treatment of HCC [4]. The combination of two cytotoxic drugs can cause more side effect than monotherapy. Therefore alternative methods using sorafenib with herbal compounds can be

better way for HCC treatment. There are several plant based compounds which showed anticancerous and antiangiogenic feature. The plant based compounds such as silymarin, silibinin, chalcones, quercetin, curcumin and epicatechins have antioxidants and anticancerous ability and are abundantly present in fruits, vegetables and various spices and have anticarcinogenic [5]. Pharmacodynamic (PD) biomarkers are indicator of treatment on the target organ in particular organism. In current scenario, protein based molecular markers have been approved which gives clear picture of drug effects. The data collected from PD biomarker studies impacts on drug development, validation and also provide pharmacologic effects of drug whether it reaches to its molecular target in expected manner[6]. PD biomarkers are good enough to highlight the drug efficacy because drug development has to move from preclinical to clinical testing. Laboratory-based approaches to predicting and evaluating potential targets are both complicated and costly [7]. Therefore, in recent years, there has been a surge of interest in using computational approaches to identify potential druggable targets early in the drug discovery and development process [8]. Direct PPIs (Protein-protein Interactions) are among the most effective indicators of a functional relationship between genes/proteins, and this information can be used to identify candidate markers in specific diseases. There are several platforms such as STRING (https://string-db.org/), Cytoscape, Database for Annotation, Visualization and Integrated Discovery (DAVID) and GEPIA (Gene Expression Profiling Interactive Analysis) to analyse and create protein – protein interaction network, functional enrichment, module development and hub gene validation. The STRING database collects, scores, and integrates all publicly available sources of protein–protein interaction (PPI) data to create a master network that predicts the biological functions of query proteins [9]. Integration of various computational platform and software to determine PPI score and enrichment analysis can validate mode of action of identified protein. Interactions between drugs and genes were predicted in the STITCH (Search Tool for Interacting Chemicals) platform on the basis of inhibition or activation of individual components, which could imply direct efficacy from chemical components at the molecular level and shed light on molecular mechanisms [10].

In the current study, we have screened potential pharmacodynamic biomarkers and subsequent we used an integrated approach to validate candidate marker involved in HCC by combining information with several bioinformatics tools.

Material & Methods

Animal model development and treatment: Animal model for HCC was successfully developed by protocol established by our research group which eliminate partial hepatectomy [11]. We have obtained approval to perform all animal experiment under the guideline of Institutional Animal Ethics Committee (503/CPCSEA). After complete development of nodules in the diseased group different doses of combinational tablets prepared by our group were given to the animal [12]. We have used formulation 2 having sorafenib and silibinin in 1: 1 ratio (4.5 mg each) with pullulan coating for sustained release of drugs.

- **Control**: Animals were orally administered saline throughout experiment.

- **Vehicle**: 12 doses of vehicle (without drug pullulan coated) tablets were given to the animals.
- **Diseased**: Single dose of DEN and 12 doses of 2-AAF were given (120 day wistar rat model established by Malik et al.,(2013) [11].
- **Treatment**: 12 doses of Sorafenib-Silibinin combinational tablets in the gap of 3 days to the 120 day old diseased model animals for 30 days.

Extraction of Sub Cellular Fraction

10% tissue (100 mg tissue in 1ml) homogenate was prepared in 0.1 M phosphate buffer containing 1.15% KCl for biochemical assay. The tissue homogenate was centrifuged at 5000 RPM for 5 min at 4^0C for removal of cell debris and nucleotide material. Post mitochondrial supernatant (PMS) was obtained after centrifugation of tissue homogenate at 10,500 RPM for 20 min at 4^0C. Both tissue homogenate and PMS was stored at -80^0C.

Biochemical Estimations:

Liver function tests: liver function tests (ALT, ALP & AST) were performed during disease progression using demonstrative units given by Span diagnostics, and the strategy was taken after as depicted by Reitman et al. [13].

Antioxidants Assays: Antioxidant assays (GSH, LPO & Catalase) were performed to analyse the ROS during hepatocarcinogenesis. Lipid peroxidation assay was performed using modified thiobarbituric acid reactive substance (TBARS) assay according to the modified Ohkwal method [14]. Total Glutathione was estimated using Jollow method [15]. Catalase activity was analyzed through Claiborne method [16].

Histopathological Examinations

The tissue slides were formed by haemotoxylin and eosin (H&E) staining and observed for histopathological changes. The images of hitopathological slides were procured by utilizing Olympus CKX41SF inverted microscope system (Olympus, Japan).

PROTEOMICS:

100 mg of liver tissue weighed and washed with PBS. The total protein extracted from tissue using Urea Lysis Buffer (Urea 8M,Thiourea 2M,CHAPS 2%, DTT 65mM, Tris base 33mM and PMSF 6mM). Tissues were incubated with ULB and for 15 min at cooling condition, later homogenized using Ploytron PT3100 homogenizer, followed by 2 stroke of sonication for 30 sec to remove DNA. Tissue debris and cell lysate were removed by centrifugation at 10,000 rpm for 15 min at 4^0C. Supernatant collected and kept at -80^0C for further analysis.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE PAGE)

Protein samples were rehydrated followed by Isoelctric Focussing (IEF) using EttanIPGphor3from GE Healthcare (USA) to immobolize the proteins on the basis of their pI value. Later, proteins were separated on SDS-PAGE gel according to molecular weight. The protein spots were visualised using silver staining. Gel imaging was done using Epson Expression 11000XL Scanner. Image Master 2D Platinum-7.0 (GE Healthcare) was used for spot detection and quantification of proteins. The controls spots were considered as reference for protein matching. Spots which were either present in one group only or demonstrated substantial changes in its expression profile as compared to control were selected for MALDI-TOF-MS analysis. The spot volume for each protein was quantified by calculation of spots intensity and area of each spot.

Identification of Differentially Expressed Proteins

Matrix-Assisted Laser Desorption and Ionization Time-of-flight Mass Spectrometry (MALDI-TOF-MS):

The trypsin digested sample was mixed with matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid in 30% Acetonitrile and 0.05% trifluoroacetic acid. The mixture was air-dried on a MALDI sample plate in triplicate. Mass spectra were obtained utilizing an Applied Biosystems Voyager-DE STR instrument. MS spectrum was generated which was corresponding to a specific peptide sequence and searched for matched peptides using MASCOT software (Matrix Science). Later the National Center for Biotechnology (NCBIprot) database was explored for *Rattus* specific taxonomy search. The multiple independently sequenced peptides from the same protein database were used to indentifyprotein and the data were checked for consistent error distribution. In Mascot, on the basis of mass value (m/Z ratio) for each peptide, probability (P) based scoring was done and similarity observed between experimental data and matched data sequence was used for protein identification.

Enrichment Analysis of Identified Proteins

All identified proteins (11 query proteins) were subjected to gene ontology analyses for molecular function, biological process, and cellular component using the online tool, Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/). Pathway enrichment was investigated using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome Pathway. A P-value of 0.05 was considered statistically significant.

Protein- Protein Interaction, Visualization and Analysis

The functional PPI examination of identified proteins was pivotal for understanding the molecular mechanisms affecting key cellular functions in carcinogenesis. Databases such as STRING, InBio-Discover, and BioGrid were used to create a primary protein–protein interaction network between the individual identified proteins (query proteins) and their neighbours. Finally, the STRING (https://string-

db.org/) platform was used to create a network of queried proteins. The master network containing query proteins and their neighbours was fed into Cytoscape to analyse protein subcellular localization, tissue expression, and drug target information. The confidence score rank from 0 to 1, with 1 being the highest possible confidence. To obtain true interactions between nodes we have use a confidence cut of 0.7. Molecular Complex Detection (MCODE) through Cytoscape software has been used to screen modules within the PPI network with MCODE scores greater than three and a number of nodes greater than five used as cut off criteria with default parameters (degree cut-off = 2, node score cut-off = 0.2, K-core = 2 and max depth = 100). To investigate the bio-molecular pathways, each cluster was subjected to an enrichment analysis. Subsequently we examined closeness centrality, network topology, network degree, and clustering coefficient in through CytoHubba plug-in. For further validation, the top 10 genes from each cluster were chosen based on their betweenness score to highlight key proteins in the interactome.

Hub Gene Validation

The Cancer Genome Atlas (TCGA) database, created in collaboration between the National Cancer Institute and the National Human Genome Research Institute, has provided extensive, multidimensional maps of the crucial genomic changes for a wide range of cancers. Gene Expression Profiling Interactive Analysis (GEIPA) (http://gepia.cancer-pku.cn/), a validated database, developed to depict TCGA expression and clinical data. Only query proteins from each cluster were selected for Hub gene validation.

Drug-gene Interaction and Visualization

The validated hub gene and both drugs sorafenib and silibinin were fed into the STITCH platform to understand relationship between drug and identified protein.

Results

Histopathological Examinations

Histopathological analysis of control, diseased, and drug combination were done after H*and*E staining. The control group has normal architecture of hepatocytes containing well-maintained central and portal vein. In diseased group a distinct hepatocellular adenoma with basophil and pale stained and vacuolated hepatocytes were seen in diseased group, which was clear indication of tumorigenic area in liver tissue. Interestingly, drug combination treated group had not showed any sign of adenoma in tissue, but vacuolated cells with disturbed hepatocyte architecture was observed as compared to diseased group (Fig. 1A-F).

Estimation of Biochemical Parameters

Liver function tests: The level of alkaline phosphatase in serum was estimated in diseased as well as treatment conditions. In control group the normal ALP level was 43.392 U/L, whereas this level significantly ($P < 0.001$) increased in diseased condition by 185.722 U/L. After 12 Doses of Combination of SF: SL, this level reduced by 130.176 U/L ($P < 0.05$). The After 30th day ALP level decreased further by

59.644 U/L ($P < 0.05$) (Fig. 1G). The level of aspartate transaminase (AST) and alanine transaminase (ALT) in serum were estimated in control, diseased as well as treatment conditions. In control group normal AST and ALT level were 15.92 U/L and ALT 11.49 U/L receptivity. This level were increased significantly ($P < 0.001$) in diseased condition to 60.99 and 44.64 U/L respectively. After treatment with SF: SL Combination tabllets AST level reduced by 19.130U/L ($P < 0.05$). Similarly, ALT level also reduced by 10.608 U/L respectively. The ALT level remained at normal condition after treatment, however AST level increased marginally with combination treatment on 30th day (Fig. 1H and I).

Antioxidants Assays: In control group MDA level was observed 1.05 nmole/hr/mg tissue, whereas this level significantly increased to 5.3 nmole/hr/mg tissue ($P < 0.001$) in diseased group. Formulations treatment group exhibited significant reduction in MDA level to 3.52 nmole/hr/mg tissue ($P < 0.01$) (Fig. 1J). Catalase activity was estimated using decomposition of H_2O_2 by catalase enzyme. In control group catalase activity was 29.45 9 nmol /min/mg protein. Whereas this level decreased by 6.95 9 ($P < 0.01$) nmol /min/mg after 4 month of DEN and 2AAF treatment. Combinational treated group showed significant restoration of catalase activity by 18.179 nmol H2O2consumed/min/mg protein (Fig. 1K). Total glutathione level was measured using DTNB method. Control group showed 0.2977 µmole GSH/gram tissues. This level diminished in disease group by 0.164 µmoleGSH/gram tissue after intoxication with single dose of DEN and 12 doses of 2-AAFTreated group showed notable normalization of GSH level by 0.02290 µmole GSH/gram (Fig. 1L).

2D SDS-PAGE Gel Analysis and Identification of Pharmacodynamic Candidate

The analysis of sample (Control, Diseased and Treatment) was done by 2D electrophoresis (Fig. 2A, B and C). It was observed through Image Master 2D Platinum software. The control gel contained 386 spots, 235 of which were unique, whereas the diseased group contained 275 spots, 125 of which were unique, and 151 of them were matched with the control group (63 over expressed and 29 down regulated). A total of 324 spots were identified in the treatment group gel, 146 of which were unique spots, and 178 spots were differentially expressed as compared to the diseased group. According to the statistical analysis of matched protein spots, 56 protein spots were significantly (P 0.05) down-regulated after treatment. Similarly, 28 spots were over expressed as compared to the diseased group. To analyze the efficacy of the formulation treatment, the eleven signatures identified as PD biomarkers with HCC progression were checked after formulation treatment. It was observed that the levels of all the 11 differentially expressed proteins have been recorded at par with the level of proteins in control animal which is a clear indication that the tablets formulation having sorafenib: silibinin was very effective in the treatment of HCC.

Identification of Protein using MALDI-TOF-MS

Selected spots from the control and diseased group gel were subjected for trypsin digestion followed by MALDITOF-MS analysis for the identification of proteins. The acquired MS spectrum, which was generates after the joining of all peptides masses used as a fingerprint to search proteins in a database.

Eleven peptide spots were identified as differentially expressed protein in diseased condition. The names of protein with sequence coverage have been listed out in the (Fig. 2E). We have also mentioned pI, molecular weight and Uniprot ID for each spots.

Comparative spot analysis

The comparative spot analysis of identified proteins in control, diseased condition and after treatment was done on the basis of % volume. It was observed that up-regulated protein spot in diseased condition (spot 25 (IPKA_Rat) and 45(CoQ4_Rat)), which were significantly down regulated after treatment. Spot in diseased condition (Spot-22 (APOC1_Rat), spot-24 (AT5G1_Rat), spot-28 (RASH_Rat), spot-31 (RRAS_Rat), spo1-32 (PPR1B_Rat), spot-95 (FOS_Rat), spot-140 (DSPP_Rat) and spot-148 (PRKCH_Rat)), which were down regulated in diseased condition reached to the normal position after treatment with sorafenib : silibinin formulation treatment (Fig. 2D).

Gene Ontology and Cancer context Analysis

The online tool DAVID was used to implement the GO annotations of all those identified. According to the GO enrichment analysis, all proteins are enriched in biological processes (BP), cellular components, and molecular function. According to the GO analysis report, the majority of the up-regulated proteins (HRAS, RRas, FOS, PRKCH, APOC1, PPP1R1B, and ACOX1) were involved in metabolic processes, specifically the positive regulation of multicellular organismal processes and the immune response-regulating cell surface receptor signalling pathway. Down-regulated proteins (COQ4, CHGA and PKIA) on the other hand, found in intracellular membrane-bounded organelles, involved in negative transport regulation and single-organism intracellular transport. Overall, enrichment analysis revealed that all identified proteins were intracellular components, mostly involved in positive regulation of multicellular organismal processes, regulation of cellular metabolic processes, and phosphate-containing compound metabolism (Supplementary Data 1).

PPI networks Construction and Analysis

All identified proteins were queried in the STRING database to create PPI network. Total 141 nodes and 1520 edges were retrieved with significant enrichment score $p < 1.0e-16$ and avg. local clustering coefficient was 0.547 and Average node degree was 21.6 (Fig. 3A). The network's largest interconnected portion was then chosen to create master network by removing parallel interactions, loops, and non connected nodes. Eight of the 11 query proteins were interconnected and formed a master network, which was visualised in Cytoscape software on the STRING module. Thereafter master network have been generated using 1st interacting partners which includes APOC1, PKIA, HRAS, RRAS, PPP1R1B, ACOX1, FOS, and PRKCH (Fig. 3B). The biological enrichment analysis of master network established that most of the proteins were involved in the positive regulation of cellular process and biological processes. Similarly enrichment score of Reactome Pathways and KEGG-pathway database suggested that most of the query protein involved in pathways related to cancer especially hepatocellular carcinoma, Hepatitis and EGF/EGFR signalling pathway (Fig. 3C) Ccomplete data compiled in supplementary data 2. The

outcome of protein interaction exhibited that RRas, RasH, PRKCH, PPP1R1B, ACOX1, FOS and PIKA were interconnected through 1st neighbour and have higher role in hepatocarcinogenesis.

Hub Gene Identification

The MCODE plug-in was used to analyze sub-modulization and to remove the background proteins, wherein six modules created based on closeness values. Four of them were significant on the basis of cluster score (< 4) (Fig. 4A-E).The CytoHubba plug-in is used to identify hub genes in each cluster based on factors such as degree, centrality, betweenness, closeness, and clustering coefficient. We chose the top ten genes based on their betweenness. Later, STRING database has been used for enrichment studies and result suggested that cluster 1 plays an important role in a number of biological processes, including the transmembrane receptor protein tyrosine kinase signalling pathway (p > 7.34E-29), Signaling by EGFR (3.68E-24), and the MAPK cascade (p > 3.41E-17). Similarly, cluster 2 was significant for its involvement in cytokine production regulation (p > 7.5E-4), Positive regulation of transcription by RNA polymerase ii (p > 3.86E-13) as well as transcription regulatory activity (1.45E-15). Cluster 3 also regulates receptor tyrosine kinase signaling (4.11E-16) and is involved in cancer-related pathways (p > 6.26E-12). Finally, cluster 4 has a significantly influence cellular metabolic processes (p > 4.12E-5) (Table 1).

Table 1
Hub Gene identification and enrichment study of clusters (complete data is complied in supplementary data 3)

Cluster name	Cluster Score	Total No of Node	Total no of edges	Hub Genes	Major Biological processes	FDR value
Cluster 1	19.478	24	175	PIK3CA, EGFR, SRC, ERBB2, **HRas**, Grb2, JAK2, SOS1, ERBB3, PTPN11	Transmembrane receptor protein tyrosine kinase signaling pathway	7.34E-29
					MAPK cascade	3.41E-17
					Signaling by EGFR	3.68E-24
Cluster 2	9.714	22	102	JUN, ESR1, RSTS2, SMAD4, HDAC1, STAT3, CTNNB, SMAD3, SP1, CAV1	Positive regulation of transcription by RNA polymerase ii	3.86E-13
					RNA polymerase II transcription regulatory region sequence-specific DNA binding	1.45E-15
					Transcription factor binding	1.14E-14
Cluster 3	6.211	20	59	NRAS, MET, TP53, IGF1R, CRK, CDH1, PTPN1, KIT, **FOS**, EREG	regulates receptor tyrosine kinase signaling	4.11E-16
					cancer-related pathways	6.26E-12
					MAPK signalling pathway	2.10E-11
Cluster 4	4.824	18	41	MAPK8, ATF2, AR, RALGDS, MAP2K2, MAPK10, SIRT1, PPARG, JUNB, **RRAS**,	cellular metabolic processes	4.12E-5
					MAPK signaling	3.72E-09
					Transcription regulator activity	1.62E-07

Hub-gene Validation

It has been observed that out of 11 differentially expressed query proteins, 3 proteins were significantly involved in different modules, i.e., HRas, RRas, and FOS. Following that, all proteins were re-analyzed using KEGG pathway enrichment analysis. We used Gene Expression Profiling Interactive Analysis

(GEPIA) to confirm the differentially expressed gene expression between HCC liver tissues and normal liver tissues (P 0.01). Finally, expression profiling revealed that HRAS, RAS, and FOS are significantly over-expressed in liver cancer tumor tissue. Similarly, overall survival revealed that high levels of the above mentioned proteins were associated with a low survival rate (Fig. 4F-H and 4F'-H').

Drug-gene interaction:

The STITCH platform was used to visualise interactions between drugs (Sorafenib: Silibinin) and validated pharmacodymanic biomarkers (RRAs, HRas, and FOS). The result showed significant (p value 3.61e-07) interaction with notable clustering coefficient of 0.647, total no of 14 nodes and 42 edges. HRas was found to be significantly (p = 7.61E-10) involved in the cellular response to growth factor stimulation, RRas regulated chemotaxis (p = 7.27E-05), and FOS was found to be significantly associated with the cell surface receptor signalling pathway (0.000273) related biological process. KEGG Pathways analysis revealed HRas and FOS were significantly involved in pathways related to the cancer (p = 2.01E-08). Similarly RRas involved in Ras signalling pathway (1.18E-10).

Discussion

The present study was carried out as an effort to identify new potential biomarkers for better understanding of liver cancer treatment. In spite of various researches over the years in the area of novel chemotherapeutic agent discovery, no effective medications have been developed to date for HCC treatment. Sorafenib (FDA approved, antiangiogenic agent) has the ability to increase the overall survival among all the available treatment available [17]. Sorafenib combination therapy can give better result than other individual chemotherapeutic medication [18]. We have evaluated efficacy of pullulan coated tablets having combination of sorafenib and silibinin in 1:1 ratio. The result indicated that histopathological changes in the liver tissue with the formulation treated group have a significant improvement in the hepatocytes and exhibit less disarrangement and degeneration of hepatocytes with no adenoma as compared to the diseased group. This improvement may be exhibited the antiangiogenic ability of silibinin to decrease intracellular ROS. Additionally proliferation of normal hepatic cell was observed to be enhanced in sorafenib-silibinin formulation treated group, which suggested that increased liver regeneration can assist to protect normal hepatocytes against tumorigenic growth. In accordance to our finding we have observed similar results in silymarin pre and post treated animal model of HCC [19, 20]. The result of biochemical analysis signifies that serum ALT, AST and ALP level were notably decreased with sorafenib-silibinin formulation. Antioxidant parameters like GSH content, MDA level and catalase activity were significantly improved with both of formulation treatment due to free radical scavenge; hence prevent the hepatocellular damage caused by DEN and 2AAF. In support of our finding, Mesallamy et al reported similar result, which revelled synergistic effect of silymarin treatment against DEN induced HCC model [19]. This promising finding suggests that molecular targeting drugs may be effective against HCC. Molecular characterization of liver cancer can also provide a personalised molecular targeted therapy. Systemic drugs can alter the expression of numerous proteins in HCC

patients, and the accuracy of their expression profiles can be measured through various proteomic tools [21, 22].

In our investigation, we used 2D gel electrophoresis to perform protein profiling for determination of differentially expressed spots after treatment. Gel imaging was used for protein spot detection and quantification, later we have identified a total of 11 differentially expressed proteins with MALD-TOF-MS. Bioinformatics analysis of these biological factors is used to identify genes that are helpful in treatment. As a result, it is critical to investigate biomarkers and related regulatory pathways that influence the development and treatment of HCC. According to gene annotation analysis of biological processes on the DAVID tool, the majority of the identified proteins were associated with positive regulation of multicellular organismal processes, phosphate metabolic processes, regulation of molecular function, protein phosphorylation, and negative regulation of cellular processes. It has previously been reported that the previous mentioned processes were linked to the progression of HCC [23]. The PPI network complex was created, and 141 nodes with 1520 edges were observed. The MCODE plug-in screened the PPI network complex to identify the modules with the highest significant score. Later, the CytoHubba plug-in was used to filter the top ten hub genes in each module complex with the highest degrees of betweenness. Finally, functional enrichment analysis and the top ten hub gene list revealed the significance of three query proteins (HRas, RRas, and FOS.) in the protein network. The steps outlined above were taken to validate pharmacodynamic biomarkers in the context of sorafenib-silibinin treatment. The association of these candidate markers (HRas, RRas, and FOS) specific to HCC progression was validated by survival analysis using GEIPA.

The most important role of H-Ras is the proliferation induced by Raf-MEK-ERK kinase and PI3K/Akt dependent pathway which support cell survival in tumor cells [24]. In accordance with the previous reports, we have also observed a significant increase in H-Ras levels in diseased conditions due to the enhanced proliferation of tumorigenic animal models. According to the survival analysis of HRas, a high level of HRas expression is associated with a low survival rate in HCC patients (Fig. 4F and F'). However, formulation treatment group showed significant reduction in its level. The drug-gene analysis on the STITCH database also validated that sorafenib-silibinin combination inhibits various kinase-related pathways as well as other signalling pathways, and found to interact with HRas via the VEGFA (vascular endothelial growth factor A) protein (Fig. 5), which is a marker candidate in hepatocarcinogenesis [25]. Our findings suggested that HRas interacts directly with KDR (a VEGFA cell-surface receptor) and Raf1. Previous research found that silibinin as well as sorafenib inhibited phosphorylation of KDR and activation of RAF1 in HCC cell lines, indicating anti-metastasis properties of both compounds [26–28]. Therefore, as a pharmacodynamic biomarker, HRas expression could indeed be used to predict the efficacy of this combination treatment.

Ras proteins switch between active GTP-bound and inactive GDP-bound states, acting as signalling switchers [29]. RRas levels are closely associated with metastasis, ErbB related pathway and have critical role in RRAS criteria in predicting HCC recurrence [30, 31]. In line with previous reports, we found a significant increase in R-Ras levels in diseased conditions as a result of the increased cell survival in

diseased group animal. To back up our findings, we ran an over-all survival analysis of RRas gene, which also indicates that over expressed RRas have been found in HCC patients Fig. 4G and G'). The formulation treatment group, on the other hand, showed a significant reduction in its level. The STITCH database revealed that RRas has direct interactions with PIK3CA (Phosphoinositide-3-kinase catalytic subunit alpha) and Raf1 (Fig. 5), which are candidate proteins for proliferation and invasion of HCC [32, 33]. Recent studies that both sorafenib and silibinin represented were found to inhibit ErbB (Ras/Raf/MEK/ERK and, PI3K/Akt/mTOR) family pathway [34]. Similarly, RRas has a direct link with NRP1 (neuropilin 1, a membrane-bound receptor) (Fig. 5), which was found to interact with VEGFs and other proangiogenic heparin-binding cytokines and can be inhibited by sorafenib [35]. As a result, RRas could also serve as a pharmacodynamic biomarker for sorafenib:silibinin combination therapy.

The role of c-Fos (also known as FOS) in HCC development is evident. C-Fos appears to be important for cell migration tumor cell proliferation in human HCC cell lines, according to research [36, 37]. Recent report also suggested that FOS expression was linked to Hepatitis B Virus (HBV) infection, alpha-fetoprotein (AFP) levels, and macrovascular invasion. In association with our research, we discovered that FOS was over expressed in disease liver tissue, though its expression was reduced after combination treatment due to the antiproliferative and antiangiogenic effects of sorafenib and silibinin. We conducted a survival analysis and boxplot on GEIPA to support our finding, which stated that higher expression is associated with a low survival rate in HCC patients Fig. 4H and H'). A Kaplan–Meier analysis by Hu et al. also observed that patients with higher FOS expression had significantly shorter median overall survival (OS) than patients with lower FOS expression in HCC patients. [38]. Drug- gene interaction on STITCH also validated that FOS has various indirect interaction with sorafenib and silibinin through number of proteins like HRAS, VEGFA, JUN (jun proto-oncogene) and PIK3CA (Fig. 5) which are key regulatory factors in hepatocarcinogenesis[39].

Concurrent with these findings, the current study demonstrated that decreased HRas, RRas, and FOS expression levels could be used to predict therapeutic intervention in HCC model.

Conclusion

In the present study, we have identified 11 candidate pharmacodynamic biomarkers through proteomic analysis. An integrated bioinformatics study based on protein-protein interaction analysis was built to analyse the hub genes and biological pathways of identified proteins. Computational analysis demonstrates that HRas, RRas, and FOS could be potential pharmacodynamic biomarkers for HCC treatment. In conclusion, we investigated the expression and therapeutic value of the above mentioned proteins with sorafenib-silibinin formulation treatment in HCC model.

Declarations

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

Dr. Ashok K. Chauhan, Founder President, Amity University Uttar Pradesh, Noida, India, provided the infrastructure for this research.

References

1. Aggarwal V, Sak K, Arora M, Iqubal A, Kumar A, Srivastava S, et al. History of Oncotherapies in Cancer Biology. Drug Targets in Cellular Processes of Cancer: From Nonclinical to Preclinical Models. Springer; 2020. pp. 1–13.
2. Sarker D, Workman P. Pharmacodynamic biomarkers for molecular cancer therapeutics. Adv Cancer Res. 2006;96:213–68.
3. Hilmi M, Neuzillet C, Calderaro J, Lafdil F, Pawlotsky J-M, Rousseau B. Angiogenesis and immune checkpoint inhibitors as therapies for hepatocellular carcinoma: current knowledge and future research directions. J Immunother Cancer. 2019;7:1–13.
4. Fornari F, Giovannini C, Piscaglia F, Gramantieri L. Elucidating the molecular basis of sorafenib resistance in HCC: Current findings and future directions. J Hepatocellular Carcinoma. 2021;8:741.
5. Ratovitski EA. Anticancer Natural Compounds as Epigenetic Modulators of Gene Expression. Curr Genomics. 2017;18:175–205.
6. Finn RS, Kudo M, Cheng A-L, Wyrwicz L, Ngan RK-C, Blanc J-F, et al. Pharmacodynamic Biomarkers Predictive of Survival Benefit with Lenvatinib in Unresectable Hepatocellular Carcinoma: From the Phase 3 REFLECT Study. Clinical Cancer Research. 2021.
7. Dogra N, Mishra S, Mani RJ, Aeri V, Katare DP. Pharmacodynamic biomarker for Hepatocellular carcinoma C: Model-based evaluation for pharmacokinetic–pharmacodynamic responses of drug. Translational Biotechnology: Elsevier; 2021. pp. 311–25.
8. Wooller SK, Benstead-Hume G, Chen X, Ali Y, Pearl FMG. Bioinformatics in translational drug discovery. Biosci Rep. 2017;37:BSR20160180.
9. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47:D607-D13.
10. Kim S. A New Computational Approach to Evaluating Systemic Gene–Gene Interactions in a Pathway Affected by Drug LY294002. Processes. 2020;8:1230.
11. Malik S, Bhatnagar S, Chaudhary N, Katare DP, Jain SK. DEN + 2-AAF-induced multistep hepatotumorigenesis in Wistar rats: supportive evidence and insights. Protoplasma. 2013;250:175–83.
12. Mishra S, Hora S, Shukla V, Das M, Kharkwal H, Katare DP. SUSTAINED RELEASE TABLETS OF SORAFENIB-SILIBININ COMBINATIONS FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA.

Int J Appl Pharm. 2018;10:117–24.

13. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957;28:56–63.
14. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–8.
15. Jollow D, Mitchell J, Zampaglione N, Gillette J. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology. 1974;11:151–69.
16. Claiborne A. Handbook of methods for oxygen radical research. Florida: CRC Press, Boca Raton.; 1985. pp. 283–4.
17. Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discovery. 2006;5:835–44.
18. Dal Lago L, D'Hondt V, Awada A. Selected combination therapy with sorafenib: a review of clinical data and perspectives in advanced solid tumors. Oncologist. 2008;13:845–58.
19. El Mesallamy HO, Metwally NS, Soliman MS, Ahmed KA, Moaty MMA. The chemopreventive effect of Ginkgo biloba and Silybum marianum extracts on hepatocarcinogenesis in rats. Cancer Cell Int. 2011;11:38.
20. Behrouj H, Ziamajidi N, Abbasalipourkabir R, Nasiri A, Asl SS. Therapeutic effect of Silybum marianum plant extract on tamoxifen-induced fatty liver in rats. Avicenna Journal of Medical Biochemistry. 2015;3.
21. Feng J, Shang S, Beretta L. Proteomics for the early detection and treatment of hepatocellular carcinoma. Oncogene. 2006;25:3810–7.
22. Zhou L, Wang K, Li Q, Nice EC, Zhang H, Huang C. Clinical proteomics-driven precision medicine for targeted cancer therapy: current overview and future perspectives. Expert Rev Proteomics. 2016;13:367–81.
23. Zhou L, Zhu W, Wang G, Cao X, Zhang X, Chen Q. Investigation of microRNA expression signatures in HCC via microRNA Gene Chip and bioinformatics analysis. Pathology-Research and Practice. 2020;216:152982.
24. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Res. 2012;72:2457–67.
25. Horwitz E, Stein I, Andreozzi M, Nemeth J, Shoham A, Pappo O, et al. Human and mouse VEGFA-amplified hepatocellular carcinomas are highly sensitive to sorafenib treatment. Cancer Discov. 2014;4:730–43.
26. Polachi N, Bai G, Li T, Chu Y, Wang X, Li S, et al. Modulatory effects of silibinin in various cell signaling pathways against liver disorders and cancer–A comprehensive review. Eur J Med Chem. 2016;123:577–95.

27. Chhabra N, Buzarbaruah S, Singh R, Kaur J. Silibinin. A promising anti-neoplastic agent for the future? A critical reappraisal. Int J Nutr Pharmacol Neurol Dis. 2013;3:206–18.
28. Asami Y, Kakeya H, Komi Y, Kojima S, Nishikawa K, Beebe K, et al. Azaspirene, a fungal product, inhibits angiogenesis by blocking Raf-1 activation. Cancer Sci. 2008;99:1853–8.
29. Zoheir KM, Abd-Rabou AA, Harisa GI, Ashour AE, Ahmad SF, Attia SM, et al. Gene expression of IQGAPs and Ras families in an experimental mouse model for hepatocellular carcinoma: a mechanistic study of cancer progression. Int J Clin Exp Pathol. 2015;8:8821.
30. He M, Qin H, Poon TC, Sze S-C, Ding X, Co NN, et al. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. Carcinogenesis. 2015;36:1008–18.
31. Roberts DE, Kakar S, Mehta N, Gill RM. A point-based histologic scoring system for hepatocellular carcinoma can stratify risk of posttransplant tumor recurrence. Am J Surg Pathol. 2018;42:855.
32. Xiao R, Shi L, Yang T, Zhang M, Wang H, Mai S. Identification of RRAS gene related to nasopharyngeal carcinoma based on pathway and network-based analyses. Translational Cancer Research. 2019;8:664–75.
33. Tornesello ML, Buonaguro L, Tatangelo F, Botti G, Izzo F, Buonaguro FM. Mutations in TP53, CTNNB1 and PIK3CA genes in hepatocellular carcinoma associated with hepatitis B and hepatitis C virus infections. Genomics. 2013;102:74–83.
34. Liang Y, Zhang T, Zhang J. Natural tyrosine kinase inhibitors acting on the epidermal growth factor receptor: Their relevance for cancer therapy. Pharmacological Research. 2020:105164.
35. Bergé M, Allanic D, Bonnin P, de Montrion C, Richard J, Suc M, et al. Neuropilin-1 is upregulated in hepatocellular carcinoma and contributes to tumour growth and vascular remodelling. J Hepatol. 2011;55:866–75.
36. Bakiri L, Hamacher R, Graña O, Guío-Carrión A, Campos-Olivas R, Martinez L, et al. Liver carcinogenesis by FOS-dependent inflammation and cholesterol dysregulation. J Exp Med. 2017;214:1387–409.
37. Watanabe T, Hiasa Y, Tokumoto Y, Hirooka M, Abe M, Ikeda Y, et al. Protein kinase R modulates c-Fos and c-Jun signaling to promote proliferation of hepatocellular carcinoma with hepatitis C virus infection. PLoS ONE. 2013;8:e67750.
38. Hu J-W, Ding G-Y, Fu P-Y, Tang W-G, Sun Q-M, Zhu X-D, et al. Identification of FOS as a candidate risk gene for liver cancer by integrated bioinformatic analysis. BioMed research international. 2020;2020.
39. Wu Z, Wei C, Wang L, He L. Determining the Traditional Chinese Medicine (TCM) Syndrome with the Best Prognosis of HBV-Related HCC and Exploring the Related Mechanism Using Network Pharmacology. Evidence-Based Complementary and Alternative Medicine. 2021;2021.

Figures

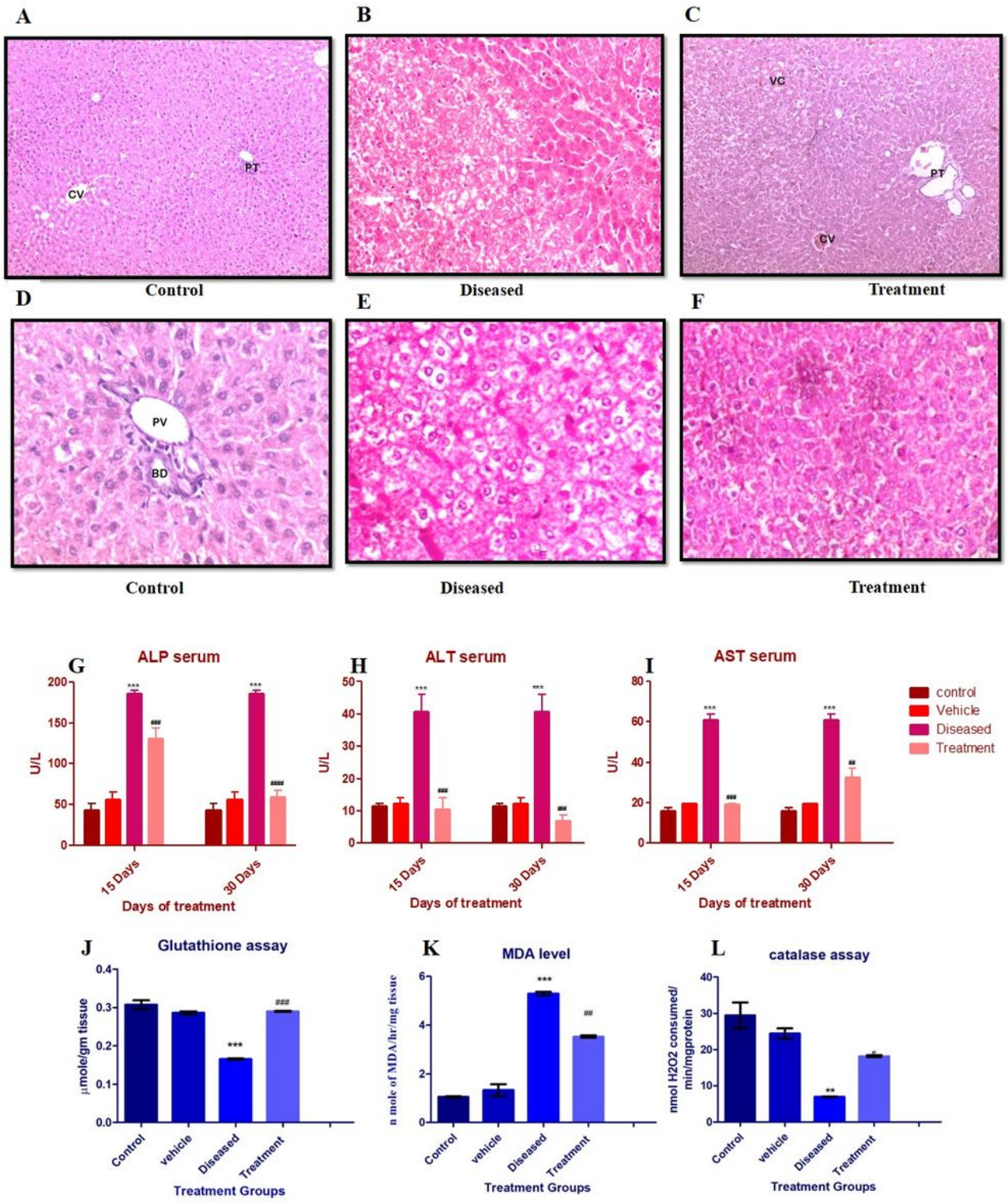
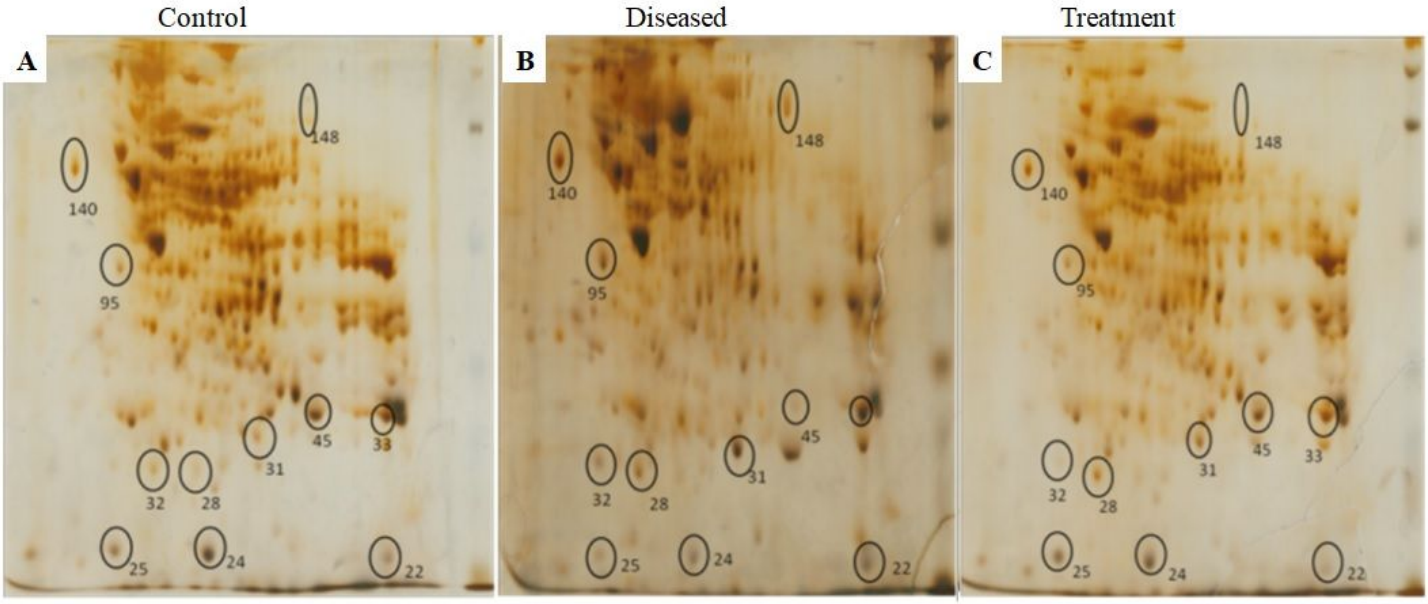
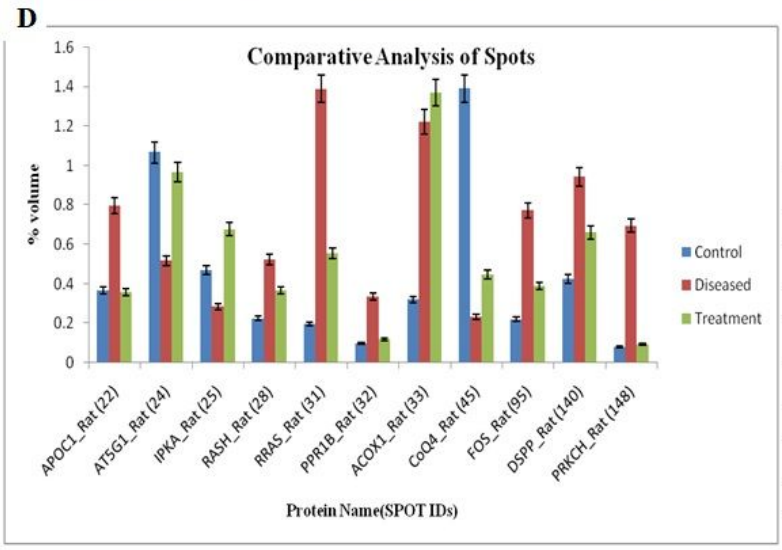


Figure 1

Histopathological changes in liver tissue (A), (B) and (C) 100X magnification of Control, Diseased, Formulation treated; (D), (E), and (F) 400X magnification of Control, Diseased and Formulation treated, respectively; Serum liver function assay after formulation treatment (G) ALP; (H) ALT; (I) AST (J)Total Glutathione activity (K) Lipid peroxidation assay, (L) Catalase activity after formulation treatment (***P<0.001 as compared to control; #P<0.05, ##P<0.01 and ###P<0.001 a Diseased versus treatment)





E

Match ID	Protein Name pI/molecular weight in kDa	Uniprot ID	Sequence Coverage	Expression profiling of Diseases spots (compared to control)
22	APOC1_Rat(9.06/7.187)	P19939	40%	Up regulated
24	CHGA_Rat (3.85/5.285)	Q06645	34%	Down regulated
25	IPKA_Rat (4.43/7.829)	P63249	33%	Down regulated
28	RASH_Rat(5.16/20.999)	P20171	42%	Up regulated
31	RRAS_Rat(6.84/23.584)	D3Z817	44%	Up regulated
32	PPR1B_Rat(4.54/22.913)	Q6J4I0	45%	Up regulated
33	ACOX1_Rat(9.10/25.002)	P07872	13%	Up regulated
45	CoQ4_Rat(7.94/26.564)	Q4FZU1	55%	Down regulated
95	FOS_Rat(4.80/40.927)	P12841	33%	Up regulated
140	DSPP_Rat(3.88/68.172)	Q62598	36%	Up regulated
148	PRKCH_Rat(7.88/81.751)	Q64617	28%	Up regulated

Figure 2

(A B and C)2D Gel of Control, Diseased and Treatment and identified 11 spots in each gel; (D) Comparative analysis of spots; (E)List of selected 11 differentially expressed proteins spots after Maldi-ToF-MS identification

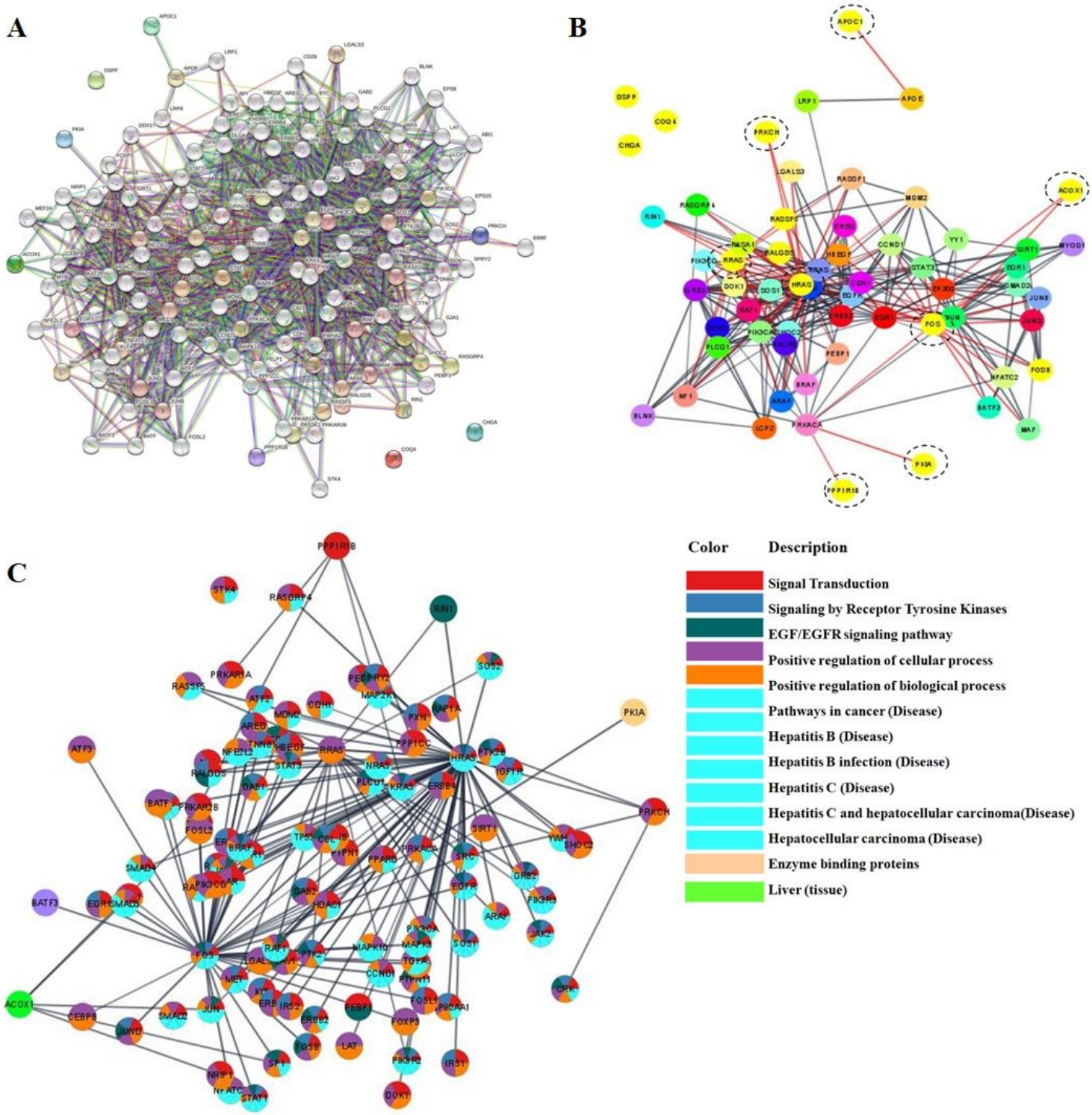


Figure 3

PPI network (A) STRING representation of network containing 141 nodes including 11 query proteins (B) Cytoscape illustration of query proteins (circled) and 1st interacting partners (C) Functional enrichment analysis of master network (cutohubba plugin) with colour coding

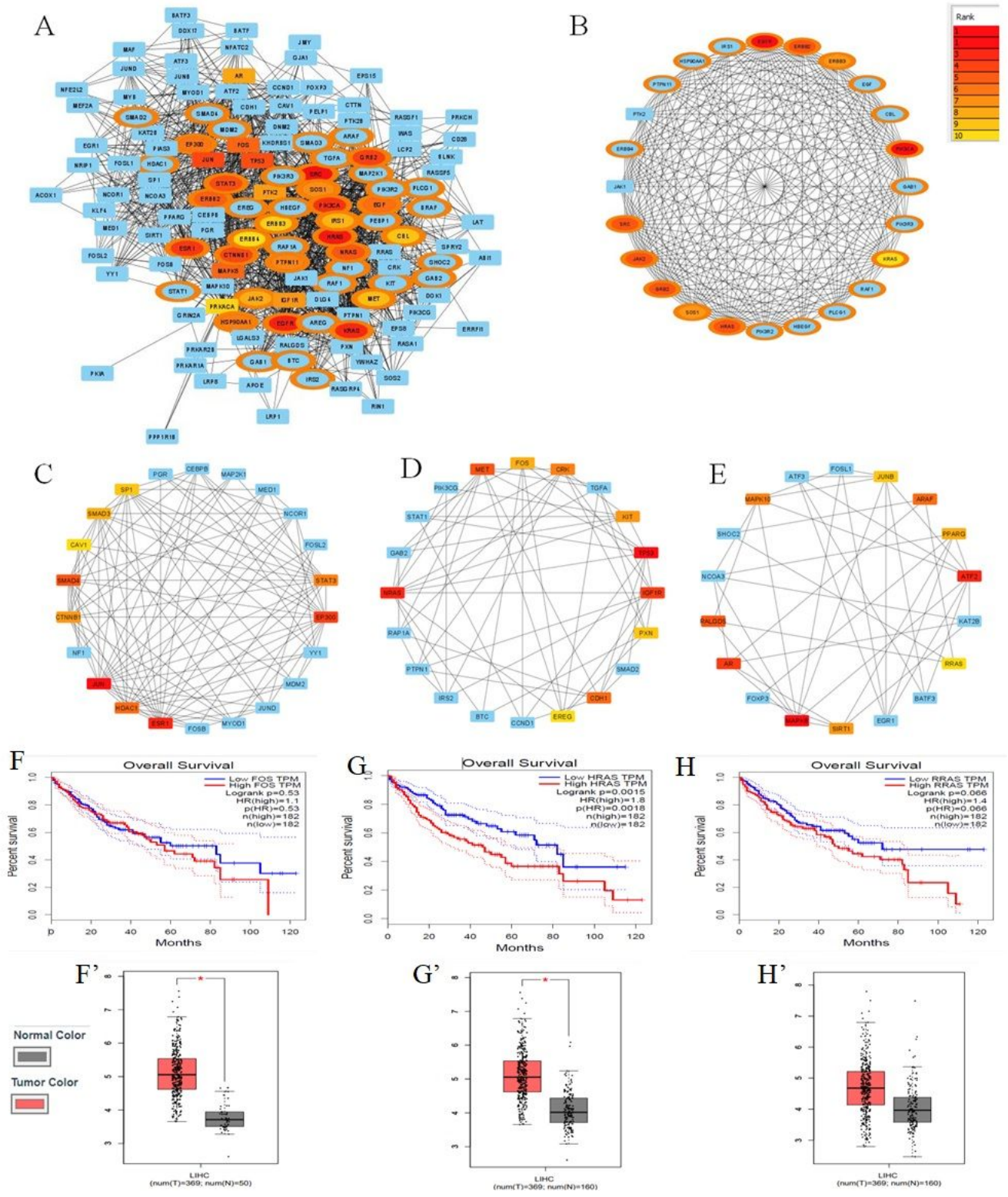


Figure 4

(A) Top 30 proteins in the master network. (B-E) cluster 1-4 respectively, (proteins were ranked on the basis of betweenness scores); GEPIA analysis(F-H)- Overall Survival analysis of selected gene in normal and liver cancer tissue : Fos (F), HRAS (G), RRAS (H) and boxplot of gene expressions in normal as well as liver cancer tissue: Fos (F'), HRAS (G'), RRAS (H') expressions

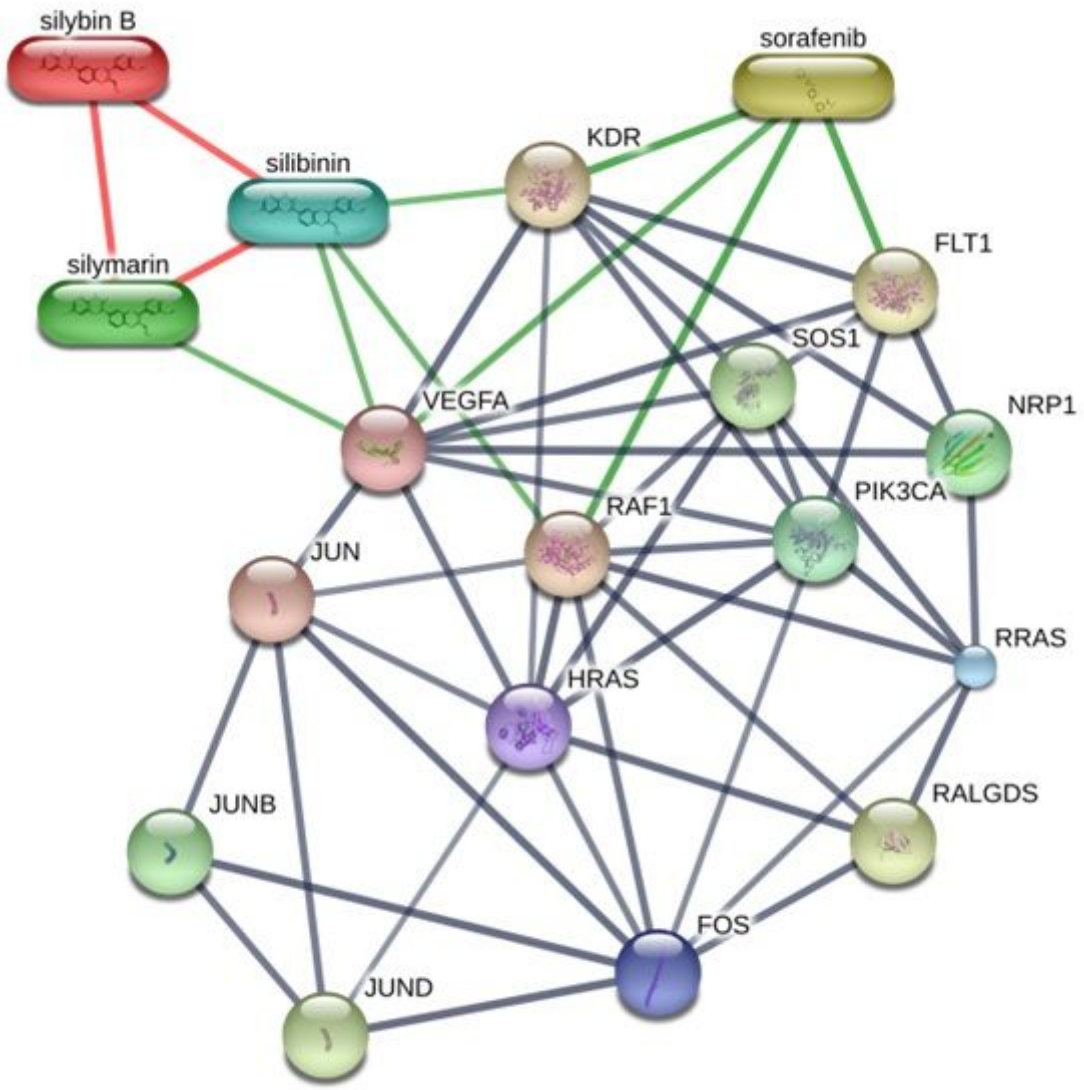


Figure 5

Drug Protein interaction network: Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryData1.xlsx
- SupplementaryData2.csv
- supplementarydata3.xlsx