Dysregulated epidermal growth factor and tumor growth factor-beta receptor signaling through GFAP-ACTA2 protein interaction in liver fibrosis

Sobia Hassan1, Zil-e-Rubab2, Hussain Shah3, Summayya Shawana4

ABSTRACT

Objective: Viral hepatitis is associated with high morbidity and mortality. Identification of biological pathways involved in hepatic fibrosis resulting from chronic hepatitis C are essential for better management of patients. Constructing the HCV-human protein interaction network through bioinformatics may enable us to discover diagnostic biological pathways. We investigated to identify dysregulated pathways and gene enrichment based on actin alpha 2 (ACTA2) and glial fibrillar acidic protein (GFAP) interaction network analysis in hepatic fibrosis.

Methods: This is an in-silico study conducted at Ziauddin University from March, 2019 to September 2019. Enrichment and protein-protein interaction (PPI) network analysis of the identified proteins: GFAP and ACTA2 along with their mapped gene data sets was performed using FunRich version 3.1.3.

Results: Biological pathway grouping showed enrichment of proteins (85.7%) in signalling pathway by epidermal growth factor receptor (EGFR) and Tumor growth factor (TGF)-beta Receptor followed by signaling by PDGF, FGFR and NGF (71.4%) (p < 0.001). SRC, PRKACA, PRKCA and PRKCD were enriched in both EGFR and TGF-beta Signalling pathways.

Conclusion: EGFR and TGF-beta signalling pathways were enriched in liver fibrosis. SRC, PRKACA, PRKCA and PRKCD were enriched and differentially expressed in both EGFR and TGF-beta signalling pathways.

KEYWORDS: ACTA2, GFAP, Liver fibrosis, Signalling by EGFR, TGF-beta receptor Signalling.

doi: https://doi.org/10.12669/pjms.36.4.1845

How to cite this:
doi: https://doi.org/10.12669/pjms.36.4.1845

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INTRODUCTION

Hepatic fibrosis is the basic damage resulting from chronic hepatitis C (CHC) which is one of the prime health challenges.1 The ultramicroscopic changes occurring in hepatic fibrosis include activation of hepatic stellate cells (HSCs) which is triggered by injury to hepatocytes.2 The excessive secretion of collagen by activated HSCs induces hyperplasia and deposition of extracellular matrix (ECM), which ultimately leads to liver fibrosis and cirrhosis.3,4 When HSCs trans differentiate into proliferative, and contractile myofibroblasts, they express certain mesenchymal markers like alpha smooth muscle actin, encoded by Actin alpha 2-ACTA2 gene which is an isofrom of the vascular smooth muscle actin and is expressed in all stages
Acidic - there is augmented expression of Glial Fibrillary Acidic - GFAP-positive HSCs in early stages of hepatic fibrosis. In addition to ACTA2, studies have shown that there is augmented expression of Glial Fibrillary Acidic - GFAP-positive HSCs in early stages of hepatic fibrosis. The GFAP gene encodes a class III intermediate filament protein expressed specifically in astrocytes of the central nervous system and their transformation capacity is well conserved. A study in rodents reported the expression of GFAP with an increased expression in the acute response to injury in the rat, and a decreased in the chronic one. It is reported that GFAP could represent a more useful marker than Alpha smooth muscle actin (α-SMA) of early HSCs activation and may be an early indicator of hepatic fibrogenesis. Our study done in 2014 revealed strong association of GFAP with the gold standard immunohistochemical marker, ACTA2 suggesting that GFAP could be a useful indicator of early HSCs activation in CHC patients. The GFAP positive hepatic cells may be antecedents of the HSCs detected by ACTA2 or they may denote a diverse subpopulation.

Most common cause of hepatocellular carcinoma (HCC) in our country is viral hepatitis. It is vital that degree of cirrhosis is established by the clinician and risk factors for HCC are identified. Bioinformatics has enabled us to discover diagnostic biomarkers and to plan treatment modalities. In light of above facts, the purpose of this study is to identify dysregulated pathways and gene enrichment based on ACTA2 and GFAP interaction network analysis in hepatic fibrosis.

METHODS

This is an in-silico study, GFAP and ACTA2 were obtained by immunohistochemistry in previous study by one of the authors which was approved by the Ethical Review Committee (Ref. Code: 1601119ZRBIO) of Ziauddin University. The study was done from March-September 2019.

In this study, the gene expression and interaction of GFAP and ACTA2 were analysed in silico. Immunoexpression of GFAP revealed substantial association with ACTA2 (α-SMA) in previous study concluding inverse relationship of GFAP with progression of fibrosis. Hence, GFAP could be characterized as useful marker for early hepatic stellate cells activation.

Bioinformatics analysis: Enrichment and protein-protein interaction (PPI) network analysis of the identified proteins: GFAP and ACTA2 along with their mapped gene data sets was performed using FunRich: Functional Enrichment analysis tool version 3.1.3 released on March 2017 http://www.funrich.org. The enriched and depleted proteins were identified by calculating fold change for biological pathways, protein domains and site of expressions.

Interaction network analysis: In FunRich software hypergeometric test, BH and Bonferroni test were applied. Normal and Overrepresented and gene ontology (GO) functional categories, significant interactions and pathways associated with datasets were identified by using the hypergeometric test and p-value correction with the BH and Bonferroni tests. Statistical cut-off of enrichment analyses was kept as default with a p-value <0.05 after Bonferroni correction.

RESULTS

Protein-Protein Interaction (PPI) Analysis of GFAP and ACTA2: The protein–protein interaction network visualization and its analysis of GFAP and ACTA2 was performed using FunRich database. The interaction network included the biological pathway enrichment of defined proteins. The PPI network was among differentially regulated interacting proteins of potential retrieved from interaction of GFAP and ACTA2 in Fig.1. Among selected GFAP and ACTA2 interacting 44 protein genes, all had interactions with each other as shown in Fig.1. The gene mapping of GFAP and ACTA2

![Fig.1: Protein-Protein interaction (PPI) Network of GFAP and ACTA2.](image-url)
Table-I: Gene Mapping and Biological Pathways Enriched in Interaction of GFAP and ACTA2 shown in Fig.1.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein Name</th>
<th>Chromosome</th>
<th>Map location</th>
<th>Interacting Genes with GFAP and ACTA2</th>
<th>Biological Pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRKCD</td>
<td>Protein kinase C, delta</td>
<td>3</td>
<td>3p21.31</td>
<td>CREBBP; PRKCA; EP300; CDK1; PRKACA</td>
<td>Retinoic acid receptors-mediated signaling</td>
<td>p = 0.009</td>
</tr>
<tr>
<td></td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
<td>17</td>
<td>17q25</td>
<td>PRKCD; PRKCA; SRC; VIM; SMAD2;</td>
<td>Alpha6Beta4Integrin</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>PRKCA</td>
<td>protein kinase C, alpha</td>
<td>17</td>
<td>17q22-q23.2</td>
<td>PRKCD; PRKCA; SRC; PRKACA; ROCK1</td>
<td>Thromboxane A2 receptor signaling</td>
<td>p = 0.017</td>
</tr>
<tr>
<td>RC</td>
<td>SRC proto-oncogene, Non-receptor tyrosine kinase</td>
<td>20</td>
<td>20q12-q13</td>
<td>PRKCD; PRKCA; SRC; CDK1; PRKACA; PSEN1; PSEN2</td>
<td>Signalling by NGF</td>
<td>p = 0.018</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
<td>10</td>
<td>10q21.1</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA</td>
<td>Signaling by EGFR</td>
<td>p = 0.023</td>
</tr>
<tr>
<td>PRKACA</td>
<td>Protein kinase, CAMP-dependent, catalytic, Alpha</td>
<td>19</td>
<td>19p13.1</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; VIM; PRKACA; APP; SNTA1</td>
<td>TNF receptor signaling pathway</td>
<td>p = 0.043</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>17</td>
<td>17q21</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A</td>
<td>TGF-beta receptor signaling</td>
<td>p = 0.051</td>
</tr>
<tr>
<td>ACTA2</td>
<td>Actin, alpha 2, smooth muscle, Aorta</td>
<td>10</td>
<td>10q23.3</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A</td>
<td>Regulation of nuclear SMAD2/3 signaling</td>
<td>p = 0.051</td>
</tr>
<tr>
<td>CREBBP</td>
<td>CREB binding protein</td>
<td>16</td>
<td>16p13.3</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A</td>
<td>Regulation of cytoplasmic and nuclear SMAD2/3 signaling</td>
<td>p = 0.051</td>
</tr>
<tr>
<td>PRKCD</td>
<td>Protein kinase C, Delta</td>
<td>3</td>
<td>3p21.31</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A</td>
<td>ALK1 signaling events</td>
<td>p = 0.076</td>
</tr>
<tr>
<td>PRKCA</td>
<td>Protein kinase C, Alpha</td>
<td>17</td>
<td>17q22-q23.2</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A</td>
<td>ALK1 pathway</td>
<td>p = 0.082</td>
</tr>
</tbody>
</table>

Interacting proteins with their chromosomal location was shown in Table-I. The enzymes represented the major category mapped along with protein kinase C and proto-oncogenes of tyrosine kinase. The leading biological pathways associated with these interacting proteins were signalling by EGFR and TGF-beta receptor signalling as depicted in Table-I.
enriched in both EGFR and TGF-beta Signalling pathways as shown in Fig.1.

In liver fibrosis, there were divergent proteome repertoires regarding EGFR and TGF beta receptor signalling. Superimposed bar chart depicted fold comparison of the differential expression of biological pathway proteins involved in EGFR Signalling (6) against TGF Beta Receptor Signalling.

### Table-II: Heat Map Showing Differentially Expressed Proteins & their Pathways Interacting with Genes enriched in Signalling by EGFR Pathway and TGF-beta receptor signaling pathway shown in Fig 1.

<table>
<thead>
<tr>
<th>Biological pathway</th>
<th>Fold enrichment</th>
<th>P-value (Hypergeometric test)</th>
<th>Genes mapped (Signalling by EGFR Pathway)</th>
<th>Biological pathway</th>
<th>Fold enrichment</th>
<th>P-value (Hypergeometric test)</th>
<th>Genes mapped (TGF-beta receptor signaling Pathway)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF receptor (ErbB1) signaling pathway</td>
<td>4.894911</td>
<td>1.47E-05</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA; ACTA2;</td>
<td>Regulation of cytoplasmic and nuclear SMAD2/3 signaling</td>
<td>20.62231</td>
<td>1.32E-12</td>
<td>CREBBP; PRKCD; PRKCA; CAMK2A;</td>
</tr>
<tr>
<td>EGFR-dependent Endothelin signaling events</td>
<td>4.891105</td>
<td>1.47E-05</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA; ACTA2;</td>
<td>TGF-beta receptor signaling</td>
<td>20.62231</td>
<td>1.32E-12</td>
<td>EP300; SRC; PRKACA; SMAD2; SNTA1;</td>
</tr>
<tr>
<td>Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met)</td>
<td>4.875939</td>
<td>1.51E-05</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA; ACTA2;</td>
<td>Signaling events mediated by VEGFR1 and VEGFR2</td>
<td>4.864626</td>
<td>6.41E-07</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A;</td>
</tr>
<tr>
<td>Signaling by EGFR</td>
<td>55.02213</td>
<td>8.48E-11</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA;</td>
<td>Signal Transduction</td>
<td>4.891105</td>
<td>6.10E-07</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A;</td>
</tr>
<tr>
<td>Signal Transduction</td>
<td>4.464134</td>
<td>0.000291</td>
<td>PRKCD; HGS; PRKCA; SRC; CDK1; PRKACA;</td>
<td>EGFR-dependent Endothelin signaling events</td>
<td>4.891105</td>
<td>6.10E-07</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SMAD2; SNTA1; CAMK2A;</td>
</tr>
<tr>
<td>Signaling by PDGF</td>
<td>57.6263</td>
<td>5.31E-09</td>
<td>PRKCD; PRKCA; SRC; CDK1; PRKACA;</td>
<td>p38 MAPK signaling pathway</td>
<td>25.89165</td>
<td>6.78E-10</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SNTA1;</td>
</tr>
<tr>
<td>Signaling by FGF</td>
<td>47.31531</td>
<td>1.45E-08</td>
<td>PRKCD; PRKCA; SRC; CDK1; PRKACA;</td>
<td>Role of Calcineurin-dependent NFAT signaling in lymphocytes</td>
<td>36.81247</td>
<td>8.49E-08</td>
<td>CREBBP; PRKCD; PRKCA; EP300; SRC; PRKACA; SNTA1;</td>
</tr>
</tbody>
</table>
GFAP-ACTA2 Protein Interaction in Liver Fibrosis

(Fig.2) Fold Comparison for Biological Pathway of proteins involved in EGFR Signalling (6) against TGF Beta Receptor.

(9). The proteins in related to EGFR signalling pathways were enriched up to 150 fold while proteins in EGFR signalling were depleted more than 130 fold (Fig.2).

Differential Expression Genes/Proteins and their Pathways: In Table-II, deep red boxes showed significant enriched pathways were Signaling by EGFR with fold enrichment of more than 2 folds and p-value: 1.51E-05 and TGF-beta receptor signalling with fold enrichment of more than 10 folds and p value: 1.32E-12. The common genes related to these pathways are PRKCD; PRKCA; SRC; CDK1; PRKACA, CREBBP; PRKCD; PRKCA; CAMK2A; EP300; SRC; PRKACA; SMAD2; SNTA1. Moreover, functional enrichment analysis of GFAP and ACTA2 interacting proteins showed 85.7% enrichment of proteins in signaling pathways of EGFR. This led to identification of another pathway, the epidermal growth factor receptor (EGFR or ErbB1) signaling system, which seems to be strongly associated with the interacting proteins GFAP and ACTA2. This finding may be due to the facilitation of crosstalk between signaling pathways by EGFR, resulting in release of various mediators of inflammation and repair.\cite{11} The EGFR signaling is reported to be a key element in not only fibrosis but also the proliferation of fibrotic liver injury to neoplastic transformation.

The study by Yang et al. has shown that EGFs can stimulate proliferation of hepatic stellate cells, which is the primary effector cell, orchestrating the deposition of extracellular matrix (ECM) in fibrotic liver.\cite{16} EGFR showed signaling enrichment of proteins similar to those in TGF\(_{\beta}\), including PRKACA, PRKCA, SRC, SMAD2 and PRKCD. Protein kinase C (PKC) is a group of calcium dependent proteins which regulate embryonic development. Various members of this PKC family have been implicated in progression of cell cycle, apoptosis and differentiation.\cite{21} Protein kinase A family of proteins is activated in response to G coupled protein receptors while PRKCD plays a key role in autophagy suppression which is achieved by the process of phosphorylation of AKT which further activates mTOR, specific for fibrolamellar carcinoma.\cite{23} In current study, activity of c-SRC

DISCUSSION

Fibrosis is a characteristic feature of end-stage liver disease and it constitutes a predominant cause of global rise in mortality and morbidity.\cite{1} Chronic hepatic injury irrespective of cause is characterized by hepatic stellate cells (HSCs) activation, proliferation, and migration within liver tissue.\cite{15} These HSCs express various mesenchymal markers upon activation\cite{9}. Expression of two such markers ACTA2 and GFAP has been demonstrated in our previous study by using immunohistochemistry.\cite{9} The management of hepatic fibrosis still remains a challenge therefore the identification of these proteins and their interacting pathways involved is critical in facilitating early diagnosis and designing target therapeutic modalities.\cite{16,17}

The Pathway analyses play a vital role understanding biological mechanisms underlying various disease processes. Therefore, they can help in identifying more potent biomarkers using dysregulated pathways.\cite{13} We used a network-based method to ascertain the dysregulated pathways elaborated in hepatitis C which may build new insights into pathogenesis of liver fibrosis.\cite{18} TGF-\(\beta\)/Smad signaling pathway is known to be one of the key fibrogenic and inflammatory pathways in the liver.\cite{19} TGF-\(\beta\)\(_1\) have been implicated in the process of activating HSCs with the magnitude of fibrosis being in proportion to increase in TGF \(\beta\) levels. Studies have shown that ACTA2 is associated with TGF \(\beta\) pathway that enhances contractile properties of HSCs leading to fibrosis.\cite{20} The results of our study show that biologic pathways associated with GFAP and ACTA2 were signaled by TGF \(\beta\) receptor signaling which is consistent with the previous studies. On the basis of close interaction of proteins, we used PPI networks to identify disease-specific networks. Our study showed a number of proteins enriched in TGF signaling primarily involving PRKACA, PRKCA, CAMK2A, SRC, SMAD2, PRKCD, CREBB and SNTA1. Moreover, functional enrichment analysis of GFAP and ACTA2 interacting proteins showed 85.7% enrichment of proteins in signaling pathways of EGFR. This led to identification of another pathway, the epidermal growth factor receptor (EGFR or ErbB1) signaling system, which seems to be strongly associated with the interacting proteins GFAP and ACTA2. This finding may be due to the facilitation of crosstalk between signaling pathways by EGFR, resulting in release of various mediators of inflammation and repair.\cite{11} The EGFR signaling is reported to be a key element in not only fibrosis but also the proliferation of fibrotic liver injury to neoplastic transformation.
increases with progressive liver fibrogenesis and hepatic stellate cell (HSC) activation. This finding is consistent with literature which reports that inhibition of SRC Kinase promotes HCV replication. The oncogenic properties of SRC family kinases have been reported with various studies upon role of SRC as target therapy in the treatment of idiopathic pulmonary fibrosis, systemic sclerosis and glioblastoma. However, its role in liver fibrosis progression is not yet understood. SRC along with PRKACA, PRKCA and PRKCD must be further explored to establish their role in target therapy of hepatic fibrosis in chronic hepatitis.

CONCLUSION

In this analysis, many perilous pathways and genes were identified based on protein-protein interaction of network GFAP and ACTA2. EGFR and TGF-beta Receptor Signalling pathways were found to be enriched in liver fibrosis through Protein Interaction studies. SRC, PRKACA, PRKCA and PRKCD were enriched and differentially expressed in both EGFR and TGF-beta Signalling pathways. These signalling pathways and related proteins are the potential targets for new therapeutic agents to combat liver fibrosis resulting from chronic hepatitis C.

Conflicts of interest: The authors declare no conflict of interest with regard to this work

Grant Support & Financial Disclosures: None.

REFERENCES