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# The Role of Tec Kinase Signaling Pathways in the Development of Mallory Denk Bodies in balloon cells in Alcoholic Hepatitis


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## Abstract

Several research strategies have been used to study the pathogenesis of alcoholic hepatitis (AH). These strategies have shown that various signaling pathways are the target of alcohol in liver cells. However, few have provided specific mechanisms associated with Mallory-Denk Bodies (MDBs) formed in Balloon cells in AH. The formation of MDBs in these hepatocytes is an indication that the mechanisms of protein quality control have failed. The MDB is the result of aggregation and accumulation of proteins in the cytoplasm of balloon degenerated liver cells. To understand the mechanisms that failed to degrade and remove proteins in the hepatocyte from patients suffering from alcoholic hepatitis, we investigated the pathways that showed significant up regulation in the AH liver biopsies compared to normal control livers. (Liu et al., 2015). Analysis of genomic profiles of AH liver biopsies and control livers by RNA-seq revealed different pathways that were up regulated significantly. In this study, the focus was on Tec kinase signaling pathways and the genes that significantly interrupt this pathway. Quantitative PCR and immunofluorescence staining results, indicated that several genes and proteins are significantly over expressed in the livers of AH patients that affect the Tec kinase signaling to PI3K which leads to activation of Akt and its downstream effectors.



### Keywords

Alcoholic hepatitis; TEC; Integrin; PAK6; Mallory-Denk Bodies


## Introduction

Liver injury from alcohol abuse involves both parenchymal and non-parenchymal cells including resident and recruited immune cells that contribute to liver damage and inflammation (Friedman, 2000). This can cause balloon hepatocytes and Mallory-Denk Body (MDB) formation. MDBs are composed of intracellular aggregations of misfolded


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proteins (increased β sheets) in ballooned hepatocytes (Kachi et al., 1993). They consist of abnormally phosphorylated, ubiquitinated, and cross-linked keratins 8 and 18 (K8/K18) and non-keratin components (Haybaeck et al., 2012). Three mechanisms of MDB formation (epigenetic mechanisms, shift from the 26S proteasome to the immunoproteasome and activation of Toll-like signaling) have recently been identified (French et al., 2010). The combination of these three mechanisms forms MDBs. Multiple membrane receptors that are involved in a variety of downstream responses including $Ca^{2+}$ influx, proliferation, differentiation, motility apoptosis, gene expression, actin reorganization and adhesion/migration (Chau et al., 2002) can affect these mechanisms. However, the detailed molecular events involved in liver MDB formation, especially in human liver disease development, remains undetermined.

The Tec tyrosine kinases are one of the largest families of none-receptor tyrosine kinases (nRTK) that catalyze phosphorylation of tyrosine residue of other proteins. They are distributed in both the cytoplasm and nucleus and their role is to mediate signal transmission through activation by stimulation of membrane receptors (Middendorp et al., 2003). Tec kinase is one of the members of the Tec family. It is predominantly expressed in hematopoietic cells and hepatocytes in mammals. The function of Tec in hepatocytes is not well understood. It has been suggested that Tec kinase plays a role in hepatocyte proliferation and liver regeneration (Xu et al., 2000). Members of these kinase families function downstream of the membrane receptors including cytokine receptors, lymphocyte surface antigens, GPCR (G-Protein Coupled Receptors), receptor type PTKs, TLR or Integrin, in hematopoietic cells and transduce signals leading to calcium mobilization, altered gene expression, production of cytokines and cell proliferation.

GPCRs constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. They have three subunits: G-AlphaQ, G-Beta and GN-Gamma.

RTKs (Receptor tyrosine kinases) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones (Robinson et al., 2000). Receptor tyrosine kinases have been shown not only to be key regulators of normal cellular processes but also to play a critical role in the development and progression of many types of cancer (Zwick et al., 2001). PAKs (p21-Activated Protein Kinases) are a growing family of serine/threonine protein kinases, which are activated by RTKs in response to extracellular signals and regulate cell shape and motility (Tang et al., 1998). PAKs have been reported to be the regulators of diverse cellular functions, including regulation of cytoskeletal dynamics, cell survival, apoptosis, hormone signaling, and gene transcription. They are able to promote cell proliferation (Bokoch, 2003; Hofmann et al., 2004; Kumar et al., 2006; Kumar and Vadlamudi, 2002; Wells and Jones, 2010). There are six different PAKs in mammals, divided into group I (PAK1–3) and group II (PAK4–6) based on the differences in their sequence, structure, and regulatory properties (Jaffer and Chernoff, 2002; Kumar et al., 2006).

Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain that function in cell surface adhesion and signaling (Humphries, 2000). Integrin alpha 6 subunits (ITGA6) may associate with a beta 1 or beta 4 subunit to form an integrin

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that interacts with extracellular matrix proteins including members of the laminin family (Lee et al., 1992). Integrin alpha 6 (ITGA6) beta 4 integrin may promote tumorigenesis, while the integrin alpha 6 (ITGA6) beta 1 integrin may negatively regulate erbB2/HER2 signaling (Kumar, 1998). Alternative splicing results in multiple transcript variants.

Activation of Toll-like receptor (TLR) signaling stimulates inflammatory and proliferative pathways. It has been shown that TLR signaling activation is the key element in the pathogenesis of Mallory-Denk bodies (MDBs) in mice fed DDC (Bardag-Gorce et al., 2010). However, little is known as to how TLR signaling is regulated and functions in MDB formation during chronic liver disease development. The TLR signaling pathways are up regulated in various chronic liver diseases, which produce inflammatory cytokines and chemokines to initiate the inflammatory cascade and are involved in activation of innate immunity in AH and NASH (Petrasek et al., 2013). The first systematic study of TLR signaling pathway transcript regulation in human liver biopsies with MDB formation was published by this lab (Liu et al., 2014). The results showed the up regulation of TLRs (TLR3 and TLR4) as well as transcript factors (NF-κB and IRF7, etc.) in the patient's liver. Toll-like receptor (TLR) activation results in the activation and up regulation of the protein tyrosine kinases.

Among the intracellular signaling molecules that are most crucial for innate immunity downstream of all the receptors on the cell membrane, are the cytoplasmic tyrosine kinases. These include the Src-family, the Syk family and the Tec family kinases. Previously, we have shown the over expression of the proteins such as spleen tyrosine kinase (Syk), and Protein Kinase B also known as Akt (Afifiyan et al., 2017) in the MDB forming liver cells. We have also indicated the up regulation of PI3K and mTOR in the liver cell cytoplasm in alcoholic hepatitis. The expression of Tec tyrosine kinases and their activation downstream of multiple membrane receptors as well as the genes involved in this family, were evaluated in the liver biopsies from alcoholic hepatitis (AH) patients. This was in search of the role of protein quality control in alcoholic hepatitis pathogenesis and the mechanisms of protein quality control in alcoholic hepatitis (AH) hepatocytes. MDB formation develops in the hepatocytes in alcoholic hepatitis as a consequence of the failure of the protein quality control mechanisms to remove misfolded and damaged proteins and to prevent MDB aggresome formation within the cytoplasm of hepatocytes (French et al.). Therefore, our study is focused on the identification and quantification of these proteins in the liver of patients with AH.

## Material and Methods

### Liver Biopsy specimens

Human formalin-fixed paraffin-embedded (FFPE) liver biopsies from patients who had alcoholic hepatitis were obtained from Harbor UCLA hospital archives as well as clinical trial funded by NIH/NIAAA grant "alcoholic hepatitis pathogenesis as determined from human liver tissue analysis" exempted as determined by the IRIS system. In all the cases, MDBs had formed in the liver. Normal livers were used for controls. The liver biopsies had been used in previous studies (French et al., 2012; Liu et al., 2014a; Liu et al., 2014b). Liver biopsy sections were cut 5μm thick. The study was carried out according to the principles of

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the Declaration of Helsinki and was designated as exempt by our institutional ethics review board. The data were analyzed anonymously and reported.

### RNA isolation

RNA isolation of FFPE sections from human liver biopsies was performed as previously described (Afifiyan et al., 2017). Briefly, the paraffin-embedded tissue sections were submerged in xylene at room temperature for 1 hour changing the xylene once after 30 minutes. The samples were hydrated by washing progressively for 2 minutes in 100%, 70%, 50% ethanol, and then pure RNase-free water. After air-drying the samples on the slides, RNA isolation was processed using the Pinpoint™ Slide RNA isolation System II (ZYMO) by adding Pinpoint™ Solution directly to the tissue section and allowing the solution to dry completely at room temperature. The embedded tissue was then removed from the slide and transferred to a micro-centrifuge tube for subsequent proteinase K digestion and RNA extraction according to the manufacturer's protocol (ZYMO). DNA-free RNA was obtained with subsequent DNase I treatment following the manufacturer's recommended protocol (ZYMO). The quality and yield of the resulting total RNAs were assessed with an absorbance reading at 260 nm (A260) using a Thermo Scientific NanoDrop™ Spectrophotometer by loading 1 μl of the extracted RNA. RNA sequencing (RNA seq)

Libraries for RNA-Seq were prepared with Nugen Ovation Human FFPE RNA-Seq Multiplex System as previously described (Liu et al., 2015). Expression pattern, function enrichment and network analysis of differentially expressed genes (DEGs) were identified using the Partek software.

### Quantitative real-time PCR analysis

Synthesis of first-strand cDNAs was performed with the above mentioned total RNA (1ug), and random hexamer primers using qScript cDNA XLT cDNA Synthesis SuperMix (Quanta Biosciences, Inc.) following instructions. Real-time PCR was performed using the Fast SYBR Green Master Mix on a StepOnePlus™ Real-time PCR System (Applied Biosystems) with a primer concentration of 300 nM. Primer sequences and the related gene Accession Number are listed in Table 1. Reaction conditions consisted of 95°C for 20 sec. followed by 40 cycles of 95°C for 3 sec. 60 °C for 30 sec. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Human α-tubulin was used as controls to normalize the starting quantity of RNA. Quantitative values were obtained from the threshold PCR cycle number (CT) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its endogenous control level and the relative mRNA expression levels were analyzed using the CT method. Reaction of each sample was performed in triplicate.

### Immunohistochemical analysis

Formalin fixed, paraffin embedded tissue slides were immunohistochemically double stained for TEC, PAK6, GNG2, STAT2, SRC (Abcam Inc., Cambridge, MA), GNA15 (MyBioSource Inc., San Diego, CA, GNAI1 (BioSS Inc, Woburn, MA) and Ubiquitin (Millipore, Temecula, CA). TEC, PAK6, GNA15, GNG2, GNAI1, STAT2, and SRC were

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detected using the second antibody donkey anti rabbit Alexa fluora 488. Ubiquitin was detected with donkey anti-mouse Alexa fluora 594 (Jackson Labs, West Grove, PA). All slides were stained with the nuclear stain DAPI (Molecular Probes, Eugene, OR). The fluorescence intensity of stained proteins of interest was measured quantitatively using a 40× objective and a standard exposure time of 800ms using a Nikon 400 fluorescent microscope with three filters (FITC-green, Texas Red, and Tri-Color), and the Nikon morphometric measurement system (Liu et al., 2015). PIK3CB staining protocol was followed as in previous a publication (Afifiyan et al., 2017).

### Statistical analysis

All data were presented as the mean ±S.E.M and were representative of at least two independent experiments done in triplicate. Statistical differences were calculated using SigmaStat software, which unpaired, two-tailed student's t-test was used to compare two groups' variance and One Way ANOVA was used to analyze the variance of multiple groups. In general, a p value less than 0.05 denoted statistical significance.

## Results

Our published study on RNA sequencing (RNA-seq) analysis of genomic profiles of AH liver biopsies and the control group (Liu et al., 2015), revealed different pathways that were up regulated significantly. About 798 differentially expressed genes (DEGs) are differentiating the AH livers from normal livers. For all the genes identified, ingenuity pathway analysis (IPA) had demonstrated a total number of 11 pathways including intracellular pathways downstream of TLR signaling. Among these differentially regulated pathways, 6 including the p70S6K signaling and Tec kinase pathways were the most significantly altered canonical pathways.

Up regulated and down regulated genes in Tec signaling are listed in Table 2. The RNA-Seq analysis revealed up regulation of TEC, FYN, STAT5A, GNG11, PAK6, GNA15, GNG2, GNAI1, STAT2, PIK3CB and ATM genes, and down regulation of the FRK gene. The expression of these genes was increased 2.5 folds up to 70 fold for STAT2 and PAK6 respectively (Fig.1). Quantitative PCR also confirmed that the expressions of the Tec family members are different in the liver of alcoholic hepatitis patients compared to normal. Based on these results the genes are divided into three groups. The first group includes TEC, GNA15 and STAT2, which show a highly significant difference in the patient's liver compare to the normal control. The result shows (Fig. 2A) a significant increase in the expression of TEC (P=0.032), GNA15 (P=0.009), and STAT2 (P=0.004). The second group indicates that the expression of the genes tends to be increased in the alcoholic hepatitis patients compared to normal control livers (Fig. 2B). This group includes FYN, GNA11, PAK6, GNG2, GNAI1, PIK3CB and integrin (ITGA6). The third group (STAT5A, FRK) is involved with the genes that have not shown any change in expression (Fig. 2C).

Double Immunohistochemical staining was performed for the genes involved in Tec kinase signaling (green) and Ubiquitin (red) to evaluate the expression of these proteins in AH liver cells compare to normal controls (Fig 3). The ubiquitin antibody stained the MDBs. Overall, the results showed more fluorescence intensity in the liver section of AH patients compared

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to the normal controls. The morphometric screen hunters were then used to measure and visualize the intensity of staining in the cytoplasm of hepatic cells of the alcoholic hepatitis patients compared to the cytoplasm of normal controls. The results indicate that AH hepatocytes are stained with a significantly higher intensity for ITGA6 (Integrin α subunit, P=<0.001), PAK6 (P=0.04), PIK3CB (P=0.02) and GNAI1 (P=<0.001) compared to the normal liver cells. The morphometric quantification of fluorescent intensity showed a 2-fold or more increase in the expression of these proteins in AH liver cells (Fig. 4). Double staining with other genes does not show a significant difference in protein concentration of these genes in intact liver cells.

Analysis of genes that involve and contribute in the Tec kinase signaling pathway by quantitative PCR and immunofluorescence staining procedures confirm up regulation of the genes that have impact in the Tec kinase signaling pathway. Among those genes TEC, GNA15, STAT2, GNAI1, PAK6, PIK3CB and Integrin were significantly up regulated either by mRNA expression and/or protein expression. It has been shown that the up regulation of these proteins can activate the Tec family kinases leading to calcium mobilization, altered gene expression, cell proliferation and apoptosis (Chau et al., 2002). The immunofluorescence staining and morphometric quantification of fluorescent intensity also indicates the significant increase in the protein level of ITGA6 (Integrin α subunit) in the liver the patients with AH. It appears that the protein is stable, as we did not see a significant difference in the level of mRNA by quantitative PCR.

## Discussion

To characterize gene expression profiles associated with the remarkable MDB formation of liver pathology in AH, liver RNA samples from 3 patients with AH and 3 controls were subjected to a RNA-seq (Liu et al., 2015). Ingenuity pathway analysis of the data showed clusters of genes involved in inflammation. Intracellular signaling pathways were up regulated in AH samples. The p70S6K pathway that includes Syk family kinase was extensively reported in a previous publication (Afifiyan et al., 2017). In this study, we have focused on the Tec kinase pathway and the genes that lead to the failure of protein quality control and affect the proliferation and liver regeneration in hepatocytes of the alcoholic hepatitis patients.

TEC is the first member of the Tec family of tyrosine kinases that was cloned by Mano (Mano et al., 1990) from liver cancer cells. Tec kinases contribute to the PI3K (Phosphatidylinositol-3 Kinase)-dependent phosphorylation and activation of PLCγ isoforms to generate IP3, the concomitant induction of sustained $Ca^{2+}$ influxes, and the activation of MAPKs like JNK (Dombroski et al., 2005) which induce gene expression and transformation.

GNA15 and GNAI1 are G-alpha subunit and GNG2 and GNG11 are G-Gamma subunit of GPCR receptors. Up regulation of these genes in AH patients can activate the transcription factor NF-κB through activation of PYK2 that leads to the stimulation of PI3K. PI3K phosphorylates membrane bound PIP2 to generate PIP3. Phosphorylation and activation of Akt by PDK1 through binding of PIP3 to the Akt domain leads to activation of IKKs, and

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finally nuclear factor NF-κB-dependent transcription. Our previous publication showed the up regulation of Akt in the cytoplasm of MDB cells (Afifiyan et al., 2017).

It has been shown that PAK6 was over expressed in HCC when compared with the adjacent noncancerous liver tissue (Chen et al., 2014). In HCC samples, elevated expression of PAK6 was associated with the number of tumor nodules, and PAK6 was positively correlated with proliferation marker Ki-67. The results suggest that PAK6 over expression is involved in the pathogenesis of HCC (Chen et al., 2014). PAK6, a member of group II PAK family, has been reported to activate the JNK and NF-kB pathways under different conditions. PAK also has been shown to mediate signals from Ras through PI3K and Akt to sustain cell transformation. Ras is become activated through RTK and spleen tyrosine kinase (Syk).

Extracellular binding of cytokines or growth factors induce activation of receptor-associated Janus kinases (JAK), which phosphorylate STATs (Signal Transducer and Activator of Transcription). The phosphorylated dimer is then actively transported to the nucleus. However, un-phosphorylated STAT proteins also shuttle between the cytosol and nucleus, and play a role in gene expression. Seven STAT proteins have been identified, STAT1 to 6, including STAT5A and STAT5B, which are encoded by distinct genes. In AH patients, mRNA of STAT2 is up regulated significantly. JAK-STAT signaling appears to be an early adaptation to facilitate intercellular communication that has co-evolved with myriad cellular signaling events.

Our results showed that there is a significant difference of ITGA6 (Integrin) expression in hepatocytes of AH patients compare to normal controls. There has been a substantial amount of research on alcohol-induced liver hypoxia in experimental and clinical alcoholic liver disease (ALD) for the past 25 years. The data has repeatedly supported a role for hypoxia in the pathogenesis of ALD but little attention has been given to this phenomenon in a clinical setting where intervention strategies could be developed. Liver hypoxia, particularly when blood alcohol levels are high, has been documented in vivo in rats fed ethanol continuously at a constant rate for prolonged periods (French, 2004) and ITGA6 is directly regulated by hypoxia inducible factors which enriches cancer stem cell activity and invasion in a metastatic breast cancer model (Brooks et al., 2016). ITGA6 is the alpha subunit of Integrin which is a member of the transmembrane heterodimer receptor family. This transduces external signals into cells through reaction with the extracellular matrix and is crucial to cell survival, migration, and differentiation. Researchers have found that the differentiation of mesenchymal stem cells (MSCs) depends on integrin related signaling pathways, including MAPK, Wnt and PI3K signaling pathways (Kligys et al., 2012; Shan and Zhang, 2017). However, there are many factors affecting the expression and activation of integrin.

Src family kinases initiate intracellular signaling in response to ligation receptor (Gauld and Cambier, 2004; Palacios and Weiss, 2004). Quantitative PCR result shows the up regulation of the expression of Src gene. Syk family kinases are activated downstream of Src kinases. Our previous publication (Afifiyan et al., 2017) showed over expression of Syk in MDB cells compare to neighboring liver cells. Activation of Tec family kinases occur downstream of both Src and Syk family kinases in the signaling pathway. These three families are the most crucial for innate immunity downstream of TLR signaling. TLRs are expressed in

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hepatic stellate cells, and hepatocytes and induce potent innate immune responses in these cell types. The liver is constantly exposed to pathogen-associated molecular patterns (PAMPs), such as LPS and bacterial DNA through bacterial translocation. The development of ethanol-induced liver injury including liver cirrhosis and severe alcoholic steatohepatitis (ASH) is a complex process involving various liver cell types and mainly factors released under the control of the innate immune system. Chronic ethanol consumption induces oxidative stress and production of reactive oxygen species (ROS), cytokine release, mitochondrial dysfunction, endoplasmic reticulum stress, and other effects. Production of reactive oxygen species (ROS) is central to the progression of many inflammatory diseases. The ROS are produced by cells that are involved in the host-defense response, such as polymorphonuclear neutrophils (PMNs). They promote endothelial dysfunction by oxidation of crucial cellular signaling proteins such as tyrosine phosphatases. The ROS act as both a signaling molecule and a mediator of inflammation.

To continue this study on the mechanism of balloon degeneration and MDB formation in hepatocytes, analysis of the Tec family tyrosine kinases and the genes that facilitate its activation were done. Figure 5 is the schematic diagram of activated Tec kinase signaling pathway which is the addition to our diagram from a previous publication (Afifiyan et al., 2017). MDB formation develops in the hepatocytes in AH patients as a consequence of the failure of the protein quality control mechanisms to remove misfolded and damaged proteins and to prevent MDB aggresome formation within the cytoplasm of hepatocytes. Based on this hypothesis, the excessive drinking of alcohol manipulates and triggers protein quality control mechanism’s failure by epigenetic phenotypic change, shift from the 26S proteasome to the immunoproteasome and activation of Toll-like signaling that forms MDBs in hepatocytes. The master regulatory target proteins that alters the mechanism of the protein quality control need to be identified and quantified. In this study detailed molecular events related to three mechanisms involved in liver MDB formation were searched for, especially in human liver disease development. Our results suggest that the Tec tyrosine kinases might be a central responsible mediator in MDB formation in ballooned cell hepatocytes. To search for the important core targets that alter the intracellular signaling pathways leading to autophagocytosis, like Syk and Akt from the previous publication (Afifiyan et al., 2017), now TEC, GNA15, GNAI1, PAK6 and STAT2 as well as ITGA6 from this study are showing that these genes might be the significant markers that need to be further evaluated. The functional effect of these proteins in the ballooned hepatocytes and MDB formation needs further investigation.

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## Abbreviation

| | |
|---|---|
| **MAPKs** | Mitogen Activated Protein Kinases |
| **JNK** | c-Jun Kinase |
| **PYK2** | Proline-Rich Tyrosine Kinase-2 |

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| | |
|---|---|
| **NF-κB** | Nuclear Factor-KappaB |
| **PIP3** | Phosphatidylinositol-3,4,5-Triphosphate |
| **IKKs** | I-κB-Kinases |
| **ITK** | (IL-2-Inducible T-cell Kinase)/EMT/TSK |
| **PI3K** | Phosphoinositide 3-Kinase |
| **STATs** | Signal Transducer and Activator of Transcription |

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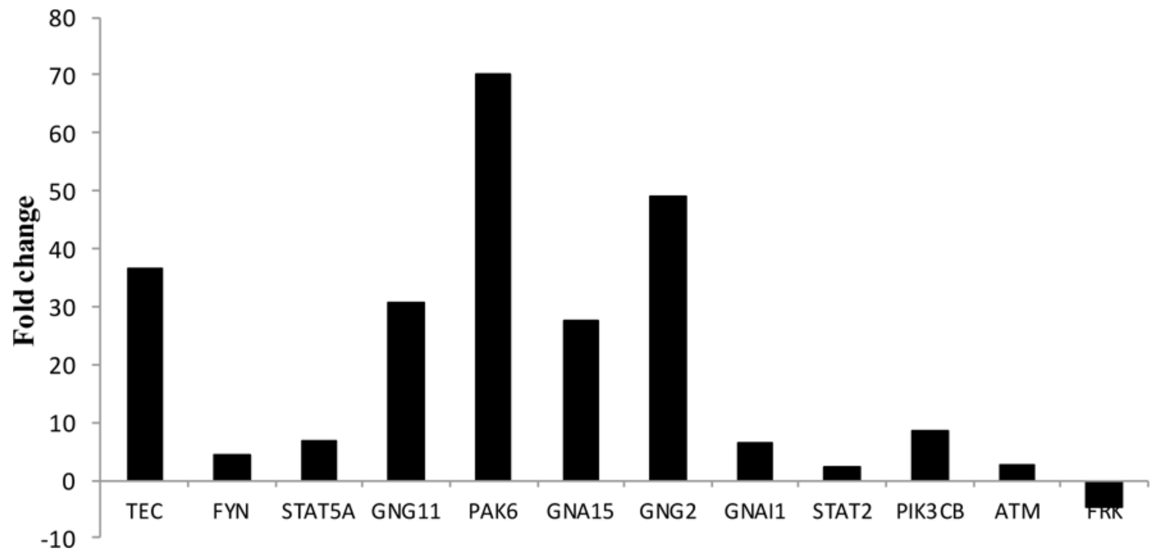


**Figure 1.**

The expression analysis of differential genes in livers of AH by RNA sequencing (Liu et al., 2015) shows the up regulated and down regulated genes in Tec kinase signaling pathway.

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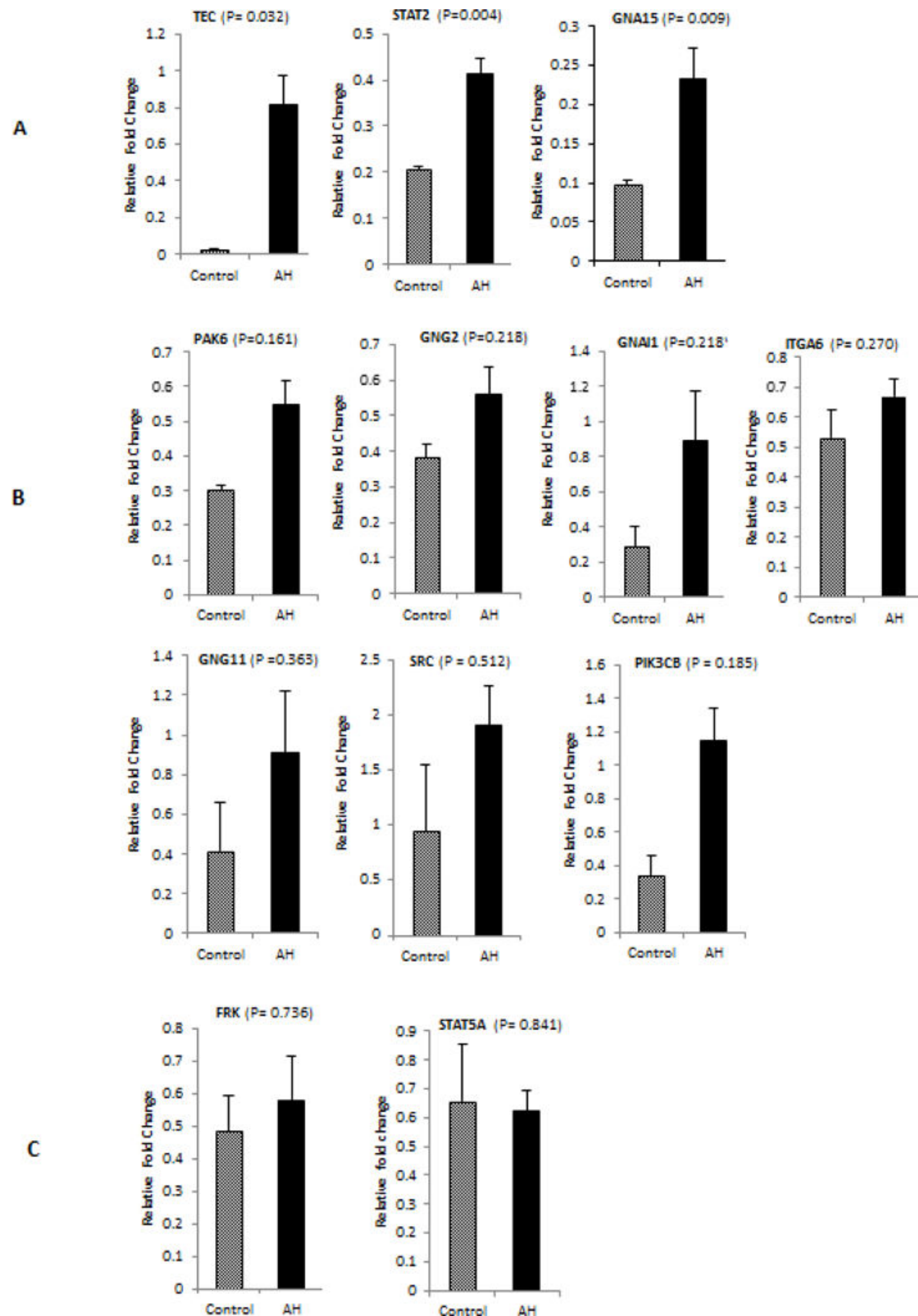


**Figure 2.**

Quantitative PCR results showed that the expressions of the Tec family members are different in the liver of the patients with alcoholic hepatitis compare to normal. Figure 2A indicates TEC (P=0.032), GNA15 (P=0.009), and STAT2 (P=0.004) which have the highly significant different mRNA expression in the patient's liver compare to normal control. Figure 2B indicates the expression of the genes including FYN, GNA11, PAK6, GNG2, GNAI1, PIK3CB and Integrin (ITGA6) that are tended to be increased in the alcoholic hepatitis patients compared to normal control livers. Figure 2C shows the qPCR results of

STAT5A, FRK that did not show any change in the expression. Data represent mean Values ± S.E.M.

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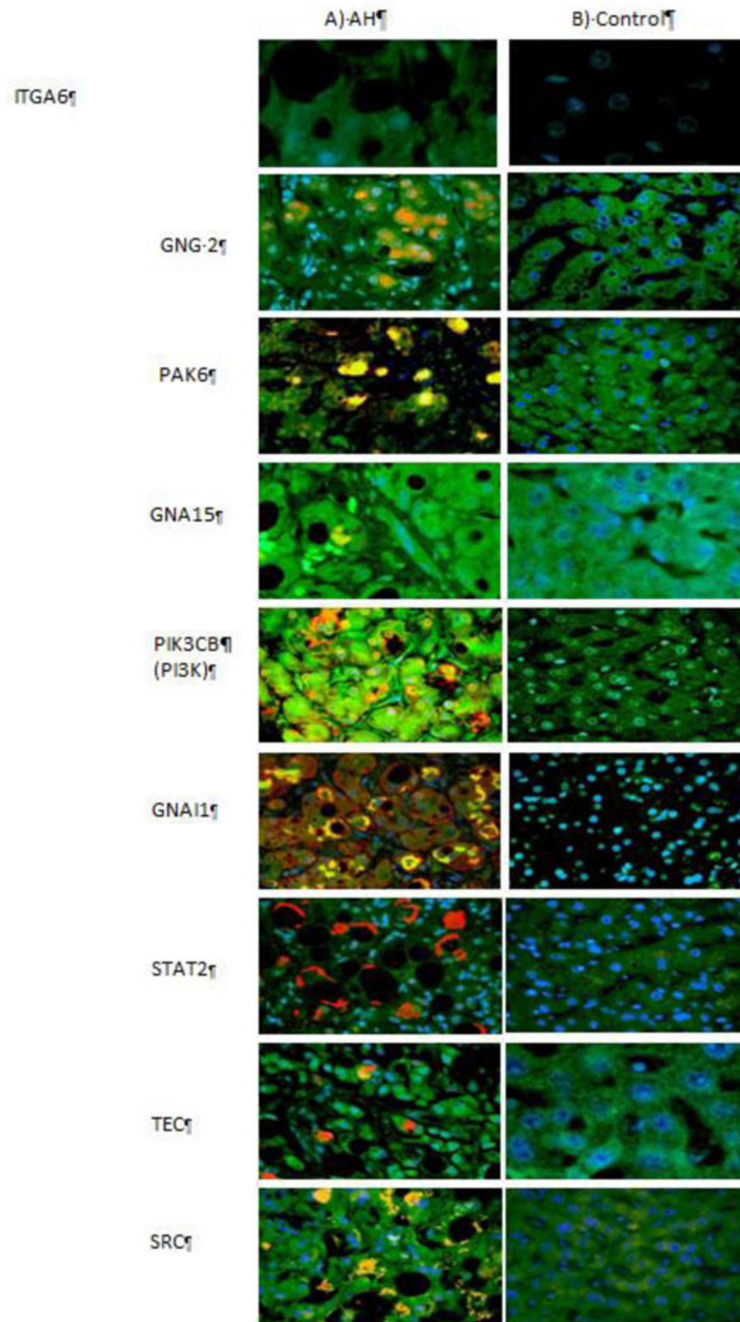


**Figure 3.**

Liver sections from different patients with AH (3A) and control (B) were double stained with antibodies to ITGA6, GNG2, PAK6, GNA15, PIK3CB, GNAI1, STAT2, TEC and SRC (green), UB (red) in the MDBs and DAPI (blue) and viewed under florescent microscope (Mag x400).

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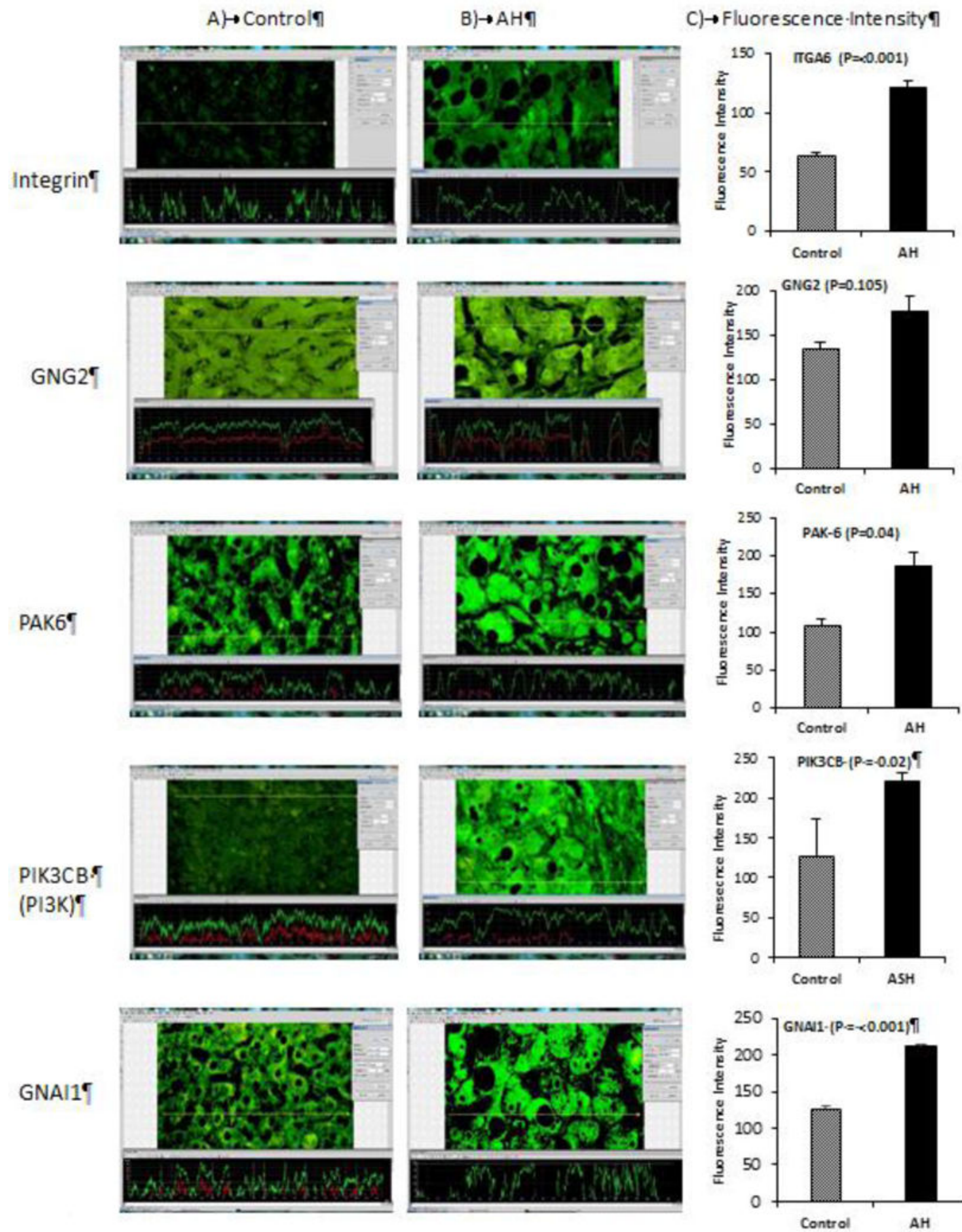


**Figure 4.**

Fluorescent intensity of ITGA6 (P=<0.001), GNG2 (P=0105), PAK6 (P=0.04), PIK3CB (P=0.02) and GNAI1 (P=<0.001) protein measured morphometrically (4C) in liver from patients (4A) with alcohol hepatitis and compared with normal control (4B) (Mag x400).

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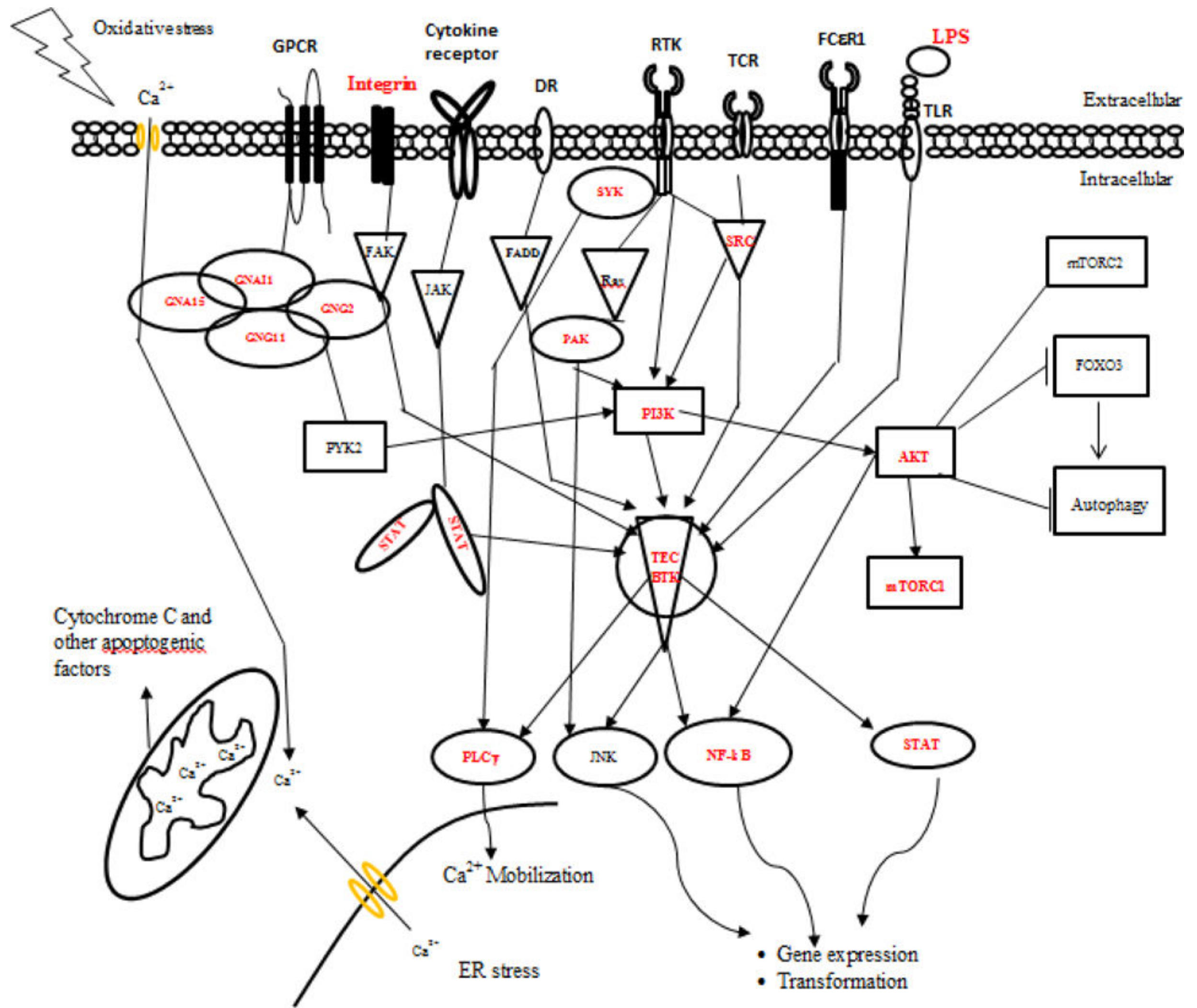


**Figure 5.**

Schematic diagram of Tec kinase signaling activation by oxidative stress and multiple membrane receptors that are involved in a variety of downstream responses including $Ca^{2+}$ influx, proliferation, differentiation and transformation.

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**Table 1**

Sequence of the forward and reverse primers used for quantitative real time PCR

| Species | Symbol | Name | Accession | Size | Sequence |
|---|---|---|---|---|---|
| Human | TUBB-1 | tubulin beta class I | NM_178014 | 196 | Forward: 5′-ACCAGGTGCTGAAAACACAT-3′<br>Reverse: 5′-CTTGAAGCTGAGATGGGAAA-3′ |
| Human | STAT5A | Signal transducer and activator of transcription 5A | NM_003152 | 151 | Forward: 5′-GAACCCTGACCATGTACTCG-3<br>Reverse: 5′-TCTGGCAGAGGTGAAAAGAC-3 |
| Human | GNG11 | guanine nucleotide binding protein (G protein), gamma 11 | NM_004126 | 74 | Forward: 5′-TTGGACCCAGTCTCAAACTT-3′<br>Reverse: 5′-CTTTTCTCAAGTCCCTGCAA-3′ |
| Human | PAK6 | p21 protein (Cdc42/Rac)-activated kinase 6 | NM_020168 | 137 | Forward: 5′-AGGGACCCATTGTTCTCTTC-3′<br>Reverse: 5′-AAGGAGACAGCAGTCTGTGG-3′ |
| Human | GNA15 | G protein subunit alpha 15 | NM_002068 | 81 | Forward: 5′-GAGAACCGCATGAAGGAGAG-3′<br>Reverse: 5′-GAGGATGACGGATGTGCTTT-3′ |
| Human | GNG2 | guanine nucleotide binding protein (G protein), gamma 2 | NM_053064 | 157 | Forward: 5′-TCAGAGCCTTAGCCACACCT-3′<br>Reverse: 5′-CCAGCTCATGGTGAATCCTT-3′ |
| Human | STAT2 | signal transducer and activator of transcription 2 | NM_005419 | 106 | Forward: 5′-CTGAAATCATCCGCCATTAC-3′<br>Reverse: 5′-TAGCACCCAAAAGCTTCATC-3′ |
| Human | GNAI1 | guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1 | NM_002069 | 299 | Forward: 5′-TTTTAGGGTGATGGCTTTGG-3′<br>Reverse: 5′-GTTGATCCAAAGGCAGGTGT-3′ |
| Human | SRC | V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) | NM_005417 | 96 | Forward: 5′-CTCTGTGGGTCTCTGGAAGA-3′<br>Reverse: 5′-GGAGTTCAGTGAGCGGAGTA-3′ |
| Human | TEC | tec protein tyrosine kinase | NM_003215 | 162 | Forward: 5′-TGTTAGCTGATGCCAGTTGA-3′<br>Reverse: 5′-TATCCAAGATGGGTTCCTGA-3′ |
| Human | PIK3CB (PI3K) | phosphoinositide-3-kinase, catalytic, beta polypeptide | NM_006219 | 123 | Forward: 5′-CCCTGCCCCATTTTATACTT-3′<br>Reverse: 5′-TTGGTGGTAATGGAAGAGGA-3′ |

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**Table 2**

Up regulated and downregulated genes in Tec kinase signaling pathway (Liu et al., 2015).

| Gene_Symbol | p-value(type) | p-value(A vs. C) | MeanRatio | FoldChange | FoldChange |
|---|---|---|---|---|---|
| TEC | 0.00052207 | 0.000522073 | 36.5385 | 36.5385 | A up vs C |
| FYN | 0.0206552 | 0.0206552 | 4.39245 | 4.39245 | A up vs C |
| STAT5A | 0.0171242 | 0.0171242 | 6.64286 | 6.64286 | A up vs C |
| GNG11 | 0.0438758 | 0.0438758 | 30.6905 | 30.6905 | A up vs C |
| PAK6 | 0.0402507 | 0.0402507 | 70.2308 | 70.2308 | A up vs C |
| GNA15 | 0.0210469 | 0.0210469 | 27.6818 | 27.6818 | A up vs C |
| GNG2 | 0.0129814 | 0.0129814 | 49 | 49 | A up vs C |
| GNAI1 | 0.00974514 | 0.00974514 | 6.4 | 6.4 | A up vs C |
| STAT2 | 0.00845362 | 0.00845362 | 2.42897 | 2.42897 | A up vs C |
| PIK3CB (PI3K) | 0.0348563 | 0.0348563 | 8.59712 | 8.59712 | A up vs C |
| ATM | 0.0336726 | 0.0336726 | 2.70231 | 2.70231 | A up vs C |
| FRK | 0.0430424 | 0.0430424 | 0.222628 | −4.4918 | A down vs C |