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Over expression of Proteins that Alter the Intracellular Signaling Pathways in the Cytoplasm of the Liver Cells Forming Mallory-Denk Bodies

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Abstract

In this study, liver biopsy sections fixed in formalin and embedded in paraffin (FFPE) from patients with alcoholic hepatitis (AH) were used. The results showed that the expression of the SYK protein was up regulated by RNA-seq and real time PCR analyses in the alcoholic hepatitis patients compared to controls. The results were supported by using the IHC fluorescent antibody staining intensity morphometric quantitation. Morphometric quantification of fluorescent intensity measurement showed a two fold increase in SYK protein in the cytoplasm of the cells forming MDBs compared to surrounding normal hepatocytes. The expression of AKT1 was also analyzed. AKT1 is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. The AKT protein was also increased in hepatocyte balloon cells forming MDBs. This observation demonstrates the role of SYK and its subsequent effect on the internal signaling pathways such as PI3K/AKT as well as p70S6K, as a potential multifunctional target in protein quality control mechanisms of hepatocytes when ER stress is activated.

Keywords

Alcoholic hepatitis; SYK; AKT1; Mallory-Denk Bodies

Introduction

To determine the mechanism of balloon degeneration and MDB formation in hepatocytes, the role of spleen tyrosine kinase (SYK), v-akt murine thymoma viral oncogene homolog 1 (AKT1), phosphoinositide-3-kinase, catalytic, beta polypeptide (PIK3CB) and the mechanistic target of rapamycin (mTOR) were evaluated in the liver biopsies from alcoholic hepatitis patients. SYK is associated with transmembrane receptors to mediate numerous signal transductions downstream of these receptors. SYK is widely expressed in

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hematopoietic cells and modulates several signaling pathways including PI3K/AKT/mTOR and p70S6K pathways. These pathways are important in regulating the cell cycle and are actively involved in mediating cell adhesions. Dysregulation of the PI3K/AKT pathway is implicated in a number of human diseases including cancer, diabetes, cardiovascular diseases and neurological diseases.

Liver injury from alcohol abuse causes balloon hepatocytes and Mallory-Denk Body (MDB) formation. MDBs are found in 70% to 75% of patients with AH (Lelbach, 1975) and many other liver diseases such as HCV (Hu and French, 1997a), , hepatocellular carcinoma, primary biliary cirrhosis, Wilson's disease and fatty liver in obesity (French, 1981), cirrhosis due to differing etiologies and hepatic adenoma (French, 1983), 2'3'-dideoxinosine (Hu and French, 1997), sclerosing hyaline necrosis in Bloom Syndrome (Wang et al., 1999) amiodarone, antitrypsin deficiency, von Gierke disease, porphyria cutanea tarda, congenital fibrosis, acute viral hepatitis and acute cholestasis (Zatloukal et al., 2007) and HBV (Nakanuma and Ohta, 1985). MDBs are composed of intracellular aggregations of misfolded proteins (increase 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).

It was previously demonstrated by our lab that in alcohol fed rats high alcohol levels inhibit the 26S proteasomes multiple catalytic activity if alcohol was fed continuously by intragastric tube feeding (Bardag-Gorce et al., 2010). We found that both the FATylation and the ufmylation protein degradation pathways were also down regulated using PCR on the liver biopsies from patients with AH, which further reduced the protein quality control in AH (Liu et al., 2014a). We have also shown that MDBs are removed by autophagocytosis (Masouminia et al., 2016a). In the search for changes in proteins within the balloon hepatocytes in alcoholic hepatitis patients, it was observed that a significant up regulation of PERK occurred, the first protein to become activated in ER stress signaling and the subsequent Unfolded Protein Response (UPR), which indicated that UPR-ER stress is induced by MDB formation in these patients (Masouminia et al., 2016b).

Our previous publication on the identification of the overall liver transcriptome using RNA sequencing showed abnormal modulation of the p70S6K signaling checkpoint in AH livers where the key candidate biomarkers (SYK, PIK3CB) were over expressed in AH patients (Liu et al., 2015). The SYK family is one out of 11 subfamilies of non-receptor type protein-tyrosine kinases (PTK) that is linked to cell surface receptors to amplify receptor activated signals inside the cell. These subfamilies are divided, based upon their functional domain and sequence motifs. SYK is a 72 KDa tyrosine kinase that was initially identified in hematopoietic cells (Mocsai et al., 2010). Studies have shown that SYK is expressed mainly in the cytoplasm of these cells and, to a small extent, in the nucleus of other cell types (Bukong et al., 2013), (Kulathu et al., 2009). The most striking unique feature of SYK is the presence of two SH2 domains (Sada et al., 2001a) that bind to the cytoplasmic region of immunoreceptors such as T cell receptors (TCR), B cell receptors (BCR), Fc receptors and NK cell receptors and plays a very important role in lymphocyte development and antigen receptor signaling. SYK is an important regulator and treatment target against hepatitis C virus infection of hepatocytes (Bukong et al., 2013). The cytoplasmic SYK is a key regulator

of signal transduction events, apoptosis and orderly cell cycle progression in B-lineage lymphoid cells (Goodman et al., 2001). It is also a candidate tumor (metastasis) suppressor that is highly expressed in mammary epithelial cells (Wang et al., 2003).

A major player that determines MDB formation is the ballooned hepatocyte. MDB-forming hepatocytes stain positive for numerous markers of preneoplastic change (French et al., 2011). These cells that have a volume increase due to hydration, form as a result of the failure of the 26S proteasome protein quality control system which leads to aggresomes composed of cytokeratins (CKs) and the misfolded proteins such as heat shock proteins (HSPs), ubiquitin (Ub), proteasome subunits, tubulin, and the ubiquitin-binding protein p62 (Yuan et al., 1996). The swelling of the balloon cell cytoplasm is due to the osmotic effect of the accumulation of these undigested proteins.

Several research strategies have been used to study the pathogenesis of 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 MDB development in AH. It is very important to identify a core target that changes the different pathways. To determine the mechanism of balloon degeneration and MDB formation in hepatocytes, the proteins that are involved in p70S6K and PI3K/AKT pathways were evaluated in these cells by RNA-seq and real time PCR analyses as well as the IHC fluorescent intensity staining morphometric system in the AH patients liver biopsies and compared to controls.

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. 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 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 of human liver biopsies was performed as we previously described (Liu et al., 2014b). Briefly, the paraffin-embedded tissue sections were mounted on a glass slide and dried at 60°C for 30 minutes. The slides were then 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 before air-drying the samples on the slides for approximately 10 minutes. 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 using a sterile scalpel followed by transferring the tissues to a micro-centrifuge tube for subsequent proteinase K digestion. The RNA was extracted and purified

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 double stained for SYK (Abcam Inc., Cambridge MA) and Ubiquitin (Millipore, Temecula, CA). A second set of slides were stained for AKT1 (Invitrogen, Camarillo, CA) and Ubiquitin. SYK and AKT1 were detected using the second antibody donkey anti rabbit Alexa fluora 488 and anti-mouse Alexa fluora 594 (Jackson Labs, West Grove, PA) respectively. Ubiquitin was detected using the second antibody donkey anti mouse Alexa Fluor 594 (Jackson Labs. West Grove, PA). All slides were stained with the nuclear stain DAPI (Molecular Probes, Eugene, OR). The fluorescence intensity of stained protein 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 system (Liu et al., 2015).

Protein extraction and immunoblotting

The total protein from biopsy sections mounted on slides was extracted with Extraction Buffer EXB using Qproteome FFPE Tissue Kit following the instructions (Qiagen, San

Diego, CA). Briefly, paraffin was first removed from tissue samples as described above. Tissue was collected from the slides and then incubated on ice for 5 min changing to 100 °C for 20 min. The tissues were then incubated at 80 °C for 2 h with agitation. After centrifugation for 15 min at 4°C, the supernatant containing the extracted proteins was transferred to a new collection tube and stored at –20°C. 50µg of each extracted protein was used to fractionate by electrophoresis using 4–20% SDS-PAGE after suspending with reducing sample buffer quickly and denaturing by heating at 100°C for 10 min. The fractionated proteins were electrophoretically transferred onto a PVDF blot membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% BSA and TBST at room temperature for 2 h and washed three times with TBST. Anti-AKT1 (Invitrogen, Carlsbad, CA) polyclonal antibody and anti β -actin monoclonal antibody (R&D systems, Minneapolis, MN) were diluted according to the company's recommendation in 3% BSA and incubated with the membranes overnight at 4 °C. After washing three times with TBST, the membranes were incubated with the corresponding secondary antibody, goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Proteins were visualized using the enhanced chemiluminescence (ECL) detection system.

Statistical analysis

All data were presented as the mean $\pm$ 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

Molecular mechanism of Mallory-Denk Body formation in ballooned hepatocytes was evaluated by using liver biopsy sections fixed in formalin and embedded in paraffin (FFPE) from patients with alcoholic hepatitis (AH) and compared with archived normal liver controls. Our published study on RNA sequencing (RNA-seq) analysis of genomic profile of AH liver biopsies and control group (Liu et al., 2015), revealed different pathways that were up regulated significantly. For all the differentially expressed genes identified, ingenuity pathway analysis (IPA) had demonstrated a total number of 11 pathways including the most significantly changed pathways. p70S6K signaling canonical pathway was among them. Up regulated and down regulated genes are listed in Table 2. The RNA-Seq analysis revealed up regulation of SYK, PIK3CB, PLCD3, CD19, IL2RG, GNAI1, SFN, PLCL1, ATM genes, and down regulation of the MAP2K1 gene. These are involved in p70S6K pathway. These genes are among 798 differentially expressed genes (DEGs) differentiating the AH livers from normal livers. Among these differentially regulated pathways, 6 pathways including the p70S6K signaling and Tec kinase pathways are the significantly altered canonical pathways that have impact on either PIK3CB and SYK or PIK3CB only expression (Table 3).

In this study, we have focused on p70S6K and PI3K/AKT/mTOR pathways and the genes that lead to the accumulation of proteins in the balloon cell cytoplasm to form multiprotein aggregates called MDBs. It was noted that expression of SYK and PIK3CB were increased 4

and 8 fold respectively by RNA sequencing analysis (Figure 1). Double immunohistochemical staining was performed for SYK (green) and Ubiquitin (red) that showed the localization of SYK in the cytoplasm of ballooned hepatocytic cells where ubiquitinated proteins were aggregated (Figure 2A to D). The morphometric screen hunters were then used to measure and visualize the intensity of SYK staining in the cytoplasm of hepatic cells with MDBs compared to the cytoplasm of neighboring normal liver cells. The results showed that the cytoplasm in hepatic cells with MDBs, stained with more intensity compared to the cytoplasm of the normal liver cells. The morphometric quantification of fluorescent intensity showed a 2 fold increase in SYK in the cells forming MDBs compared to surrounding normal hepatocytes (Figure 2E). Double staining with a mitochondrial marker clearly showed marked positive cytoplasm staining for SYK and not mitochondria staining (data not shown). Quantitative PCR of SYK (Figure 2F) showed a significant increase in the expression compared to controls ($P=0.016$)

Double immunohistochemical staining was also performed on AKT1 (green) and Ubiquitin (red), mTOR (green) and Ubiquitin (red) as well as PIK3CB (green) and Ubiquitin (red). This showed that the fluorescent intensity for these genes was significantly higher in the FFPE liver biopsy sections from patients with AH compared with normal liver controls. All these proteins have been shown to be involved in regulating cell survival, proliferation and protein synthesis, and hence cell growth (Engelman et al., 2006; Vivanco and Sawyers, 2002).

Staining with antibodies to AKT1 and Ubiquitin indicated the significant increase in the level of AKT1 in the ballooned hepatocytes compared to adjacent normal cells (Figure 3A and 3B). Similar to SYK, the morphometric screen hunters showed that the cytoplasm of hepatic cells with MDBs stained with more intensity for AKT1 compared to the normal liver cells (Figure 3D). The protein level of AKT1 in MDB-forming hepatic cells was also evaluated by Western blot (Figure 3F). A marked increase in the AH livers compared with normal controls was observed (Figure 3E). This indicates the important roles of AKT protein in MDB forming ballooned hepatocytes.

Figure 4 and 5 show the immunohistochemical staining of mTOR/Ub and PIK3CB/Ub respectively. These show that the staining intensity was increased in the liver cell cytoplasm for both proteins and also is significantly increased in the AH biopsies compared to the normal control livers. These data indicate a possible role of the PI3K/AKT/mTOR checkpoint in the reduced liver autophagocytosis observed in MDB forming ballooned liver cell and AH pathogenesis.

Quantitative PCR also confirmed that the expression of AKT1 and mTOR tended to be increased in the alcoholic hepatitis patients compared to normal control livers (Figures 2G and 3G).

Discussion

Balloon cell degeneration was first reported in patients dying from alcoholic cirrhosis (Mallory, 1911). Mallory described large hepatocytes which had cytoplasmic inclusions now

called Mallory-Denk Bodies (MDBs). In this study, we showed that the intracellular signaling pathways are dysregulated in MDB forming cells. The most important are PI3K/AKT/mTOR and p70S6k pathways, implicated in cell proliferation, survival, growth, and protein synthesis. To assess the pathogenesis of the balloon hepatocytes, which we believe is due to loss of protein quality control mechanisms, it is important to detect the proteins that are over expressed in the cytoplasm of MDB forming balloon cells, indicating that these proteins are involved in MDB formation. Here, we found for the first time, that the levels of a few proteins, such as SYK and AKT1 are increased in the MDB forming liver cells. The results also showed over expression of mTOR and PIK3CB in the cytoplasm of liver cells of alcoholic hepatitis patients. These proteins are engaged in the p70S6k and PIK3/AKT/mTOR pathways and are important in regulating the cell cycle and are actively involved in mediating cell adhesions. Dysregulation of the PI3K/AKT pathway is implicated in a number of human diseases including cancer, diabetes, cardiovascular diseases and neurological diseases.

Ethanol in hepatocytes is oxidized by the cytosolic alcohol dehydrogenase (ADH) to form acetaldehyde. Mitochondrial aldehyde dehydrogenases (ALDH2) then further catalyzes the production of acetate. Acetaldehyde is highly toxic; playing an important role in adduct formation, impairing hepatocyte secretory pathways (Thiele et al., 2008), contributing to immune responses (Viitala et al., 2000) and release of inflammatory cytokines (Tuma and Casey, 2003). Induction of the cytochrome P450 2E1 (CYP2E1) is also a key response to alcohol intake, resulting in an increased production of reactive oxidative species (ROS), mainly H₂O₂ and superoxide anion (oxidative stress, Cederbaum, 2006). In addition, Kupffer cells (Thakur et al., 2007) and infiltrating neutrophils (through NADPH oxidase 2) are also important sources of ROS. At high blood alcohol levels, NAD⁺ is converted to the reduced state (NADH) and is not available to act as a coenzyme in the deacetylation of molecules by the SIRT deacetylases (French, 2015); (Bardag-Gorce et al., 2002). Hyper acetylation of numerous regulatory proteins and NAD⁺ reduction result in accelerating the effects of ROS (Fernandez-Checa et al., 1993) leading to endoplasmic reticulum (ER) stress. The hepatocytes' endoplasmic reticulum plays an essential role in ensuring proper folding of the newly synthesized proteins. Evidence suggests that hepatocyte ER stress initially induces the compensatory over expression of viability factors, including keratins 8 and 18 (K8/18), but ultimately results in accumulation of misfolded proteins (Zatloukal et al., 2007). Ballooned hepatocytes exhibit endoplasmic reticulum stress and represent an extreme morphologic manifestation of abnormal protein turnover. This is supported by immunohistochemical characterization of these cells, which reveals loss of cytosolic K8/18 expression and accumulation of ubiquitinated aggregates of K8/18 proteins (Lackner et al., 2008). ER stress, under a severe insult, such as chronic alcohol consumption, activates protein degradation pathways to preserve cell function and viability (Bernales et al., 2006). One is autophagy which is mediated by lysosomal derived catalytic enzymes digestion and related to Ca²⁺ release from ER. Aggregates like MDBs, activate ER stress driven autophagy (Massominia et al., 2016b).

Oxidative stress activates SYK and its expression required for oxidative stress-mediated Ca²⁺ mobilization and JNK activation (Qin et al., 1997). It has been shown that SYK mediates inducing Ca²⁺ release from intracellular stores. Lack of SYK expression decreases

cell viability induced by H₂O₂ treatment in B cells (Sada et al., 2001b). Activated SYK modulates the PI3K/AKT/mTOR survival pathway to inhibit Caspase-9 activity, preventing apoptosis (Ding et al., 2000). The downstream signaling events modulated by the presence of activated SYK are still under investigation, but activation of the PI3K/AKT/mTOR pathway is a likely contributor (Figure 6).

Recent studies on SYK activation in the liver of experimental mice and human alcohol liver disease (ALD) have shown that SYK, a non-receptor tyrosine kinase, was involved in the promotion of steatosis development. It also played a modulating role in immune cell-driven liver inflammation, and hepatocyte cell death at different stages of ALD. Inhibition of SYK activation in the mouse model abrogated alcohol-induced neutrophil infiltration, resident immune cell and inflammasome activation, ERK1/2 mediated NF κ B activation and IRF3-mediated apoptosis (Bukong et al., 2016a). It has recently been shown that STING and IRF3 modulate hepatocyte cell death during ALD, thereby, linking ER stress signaling with the mitochondrial pathway. Using a mouse model of binge drinking, it was found that SYK activation was involved in modulating numerous signaling events previously linked to the development of alcohol-induced liver pathology (Bukong et al., 2016b). They found that in the alcohol-inducing SYK activation mouse model that a SYK inhibitor reduced the steatosis, inflammation, NF κ B, TNF α , and MCP-1 mRNA expression and serum ALT response to alcohol feeding (Bukong et al., 2016b). SYK is a key molecule that controls multiple physiological functions in cells including Ca²⁺ mobilization, MAPK cascades and hepatic oxidative stress (Sada et al., 2001b). SYK has tyrosine phosphorylated at 10 autophosphorylated sites. Each site performs a different function by interacting with a different molecule, i.e. LAT, VAV1, PI3K, CM, PIP, PYK2, Bik, SLP, Shc, TCR, 3BP2, Cbi, PLC γ , PLD, 13+K, MAPK, tubulin and SH3P7. SYK is a potent modulator of epithelial cell growth and a potential tumor suppressor (Wang et al., 2003). In our study, RNA sequence analysis of FFPE liver tissue from patients with alcoholic hepatitis showed SYK expression was increased 4 fold compared to control samples. Over expression of SYK was also confirmed by IHC and qPCR.

Two mTOR complexes, mTORC1 and mTORC2 are regulated by nutrients, stress and insulin/IGF-1. The mTORC1 containing raptor is a key regulator of translation and ribosome biogenesis in mammalian cells, and is responsible for autophagy induction in response to starvation. The proposed mechanisms related to Ca²⁺ release from ER which activates the CamKK/AMPK-dependent pathway (Masouminia et al., 2016b) inhibits mTOR to induce autophagy (Hoyer-Hansen and Jaattela, 2007). On the other hand, mTORC2 is involved in the regulation of phosphorylation and activation of AKT/PKB (Jung et al., 2010). It has been speculated that mTORC2 acts as a negative regulator of autophagy. Indeed, mTORC2 inhibition induced autophagy and atrophy in skeletal muscle cells under a fasting condition (Mammucari et al., 2007; Zhao et al., 2007). However, the autophagy induction caused by mTORC2 inhibition is mediated mainly by FoxO3, a transcription factor downstream of AKT that is involved in autophagy gene expression (Figure 6).

The activation of mTOR, which lies downstream of AKT, is correlated with the presence of activated SYK (Carnevale et al., 2013; Leseux et al., 2006) which can increase the level of the anti-apoptotic protein, Mcl-1 due to activation of PKC δ and the PI3K/AKT pathways

(Baudot et al., 2009; Gobessi et al., 2009; Zhang et al., 2012). Active AKT negatively regulates GSK3 to block the phosphorylation of Mcl-1, an event that triggers its ubiquitination and proteosomal degradation (Ding et al., 2007). The immunohistochemistry analysis showed that mTOR is up regulated in hepatocytes in AH (Figure 4).

In this study, the over expression of protein kinase B (PKB), also known as AKT, was noted. It is a serine/threonine-specific protein kinase. Activated AKT by PI3K, mediates downstream responses, including cell survival, growth, proliferation, cell migration and angiogenesis by phosphorylating a range of intracellular proteins (Manning and Cantley, 2007). AKT also promotes G1/S cell cycle progression upon activation. In our previous publication, it was shown that the altered modulation of the G1/S cell cycle checkpoint in AH with MDBs present could provide a new explanation for the mechanisms that are underlying the cause for cell cycle arrest and the inhibition of liver regeneration seen in AH (Liu et al., 2015; French et al., 2016).

A schematic diagram of activated p70S6K signaling pathway derived using ingenuity pathway analysis (IPA) has been published recently based on our sequencing results (Liu et al., 2015). Here, the expressions of proteins and mediators that take part in the PI3K/AKT/mTOR pathway following ER stress in the liver was schematically illustrated (Figure 6)

Over expression of SYK, PIK3CB, AKT1 and mTOR indicates the acute change in protein quality control in ballooned hepatocytes and shows dysregulation in the protein quality control. It has not been shown yet that SYK is activated in MDB forming ballooned liver cells. It is not clear that the accumulation of SYK and AKT in the cytoplasm of the ballooned cells is due to misfolding of these proteins or changes in protein stability. However, the pathogenicity of the ballooned hepatocytes might be the results of the protein quality control loss inside these cells and that could be the results of over production of ROS and the ER stress.

Over expression of AKT and SYK in the cytoplasm of the ballooned cell forming MDBs may be the important core targets that alter the intracellular signaling pathways leading to autophagocytosis. The functional effect of these proteins in the ballooned hepatocytes still needs to be further investigated.

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References

- Bardag-Gorce F, French BA, Li J, Riley NE, Yuan QX, Valinluck V, Fu P, Ingelman-Sundberg M, Yoon S, French SW. The importance of cycling of blood alcohol levels in the pathogenesis of experimental alcoholic liver disease in rats. *Gastroenterology*. 2002; 123:325–35. [PubMed: 12105860]
- Bardag-Gorce F, Oliva J, Lin A, Li J, French BA, French SW. Sema prevents the up regulation of toll-like receptor signaling in Mallory-Denk body forming hepatocytes. *Exp Mol Pathol*. 2010; 88:376–9. [PubMed: 20206621]
- Baudot AD, Jeandel PY, Mouska X, Maurer U, Tartare-Deckert S, Raynaud SD, Cassuto JP, Ticchioni M, Deckert M. The tyrosine kinase Syk regulates the survival of chronic lymphocytic leukemia B

cells through PKCdelta and proteasome-dependent regulation of Mcl-1 expression. *Oncogene*. 2009; 28:3261–73. [PubMed: 19581935]

Bernales S, Papa FR, Walter P. Intracellular signaling by the unfolded protein response. *Annu Rev Cell Dev Biol*. 2006; 22:487–508. [PubMed: 16822172]

Bukong TN, Iracheta-Vellve A, Gyongyosi B, Ambade A, Catalano D, Kodys K, Szabo G. Therapeutic Benefits of Spleen Tyrosine Kinase Inhibitor Administration on Binge Drinking-Induced Alcoholic Liver Injury, Steatosis, and Inflammation in Mice. *Alcohol Clin Exp Res*. 2016a; 40:1524–30. [PubMed: 27177528]

Bukong TN, Iracheta-Vellve A, Saha B, Ambade A, Satishchandran A, Gyongyosi B, Lowe P, Catalano D, Kodys K, Szabo G. Inhibition of spleen tyrosine kinase activation ameliorates inflammation, cell death, and steatosis in alcoholic liver disease. *Hepatology*. 2016b; 64:1057–71. [PubMed: 27302565]

Bukong TN, Kodys K, Szabo G. Human ezrin-moesin-radixin proteins modulate hepatitis C virus infection. *Hepatology*. 2013; 58:1569–79. [PubMed: 23703860]

Carnevale J, Ross L, Puissant A, Banerji V, Stone RM, DeAngelo DJ, Ross KN, Stegmaier K. SYK regulates mTOR signaling in AML. *Leukemia*. 2013; 27:2118–28. [PubMed: 23535559]

Cederbaum AI. Cytochrome P450 2E1-dependent oxidant stress and upregulation of anti-oxidant defense in liver cells. *J Gastroenterol Hepatol*. 2006; 21(Suppl 3):S22–5. [PubMed: 16958665]

Ding J, Takano T, Gao S, Han W, Noda C, Yanagi S, Yamamura H. Syk is required for the activation of Akt survival pathway in B cells exposed to oxidative stress. *J Biol Chem*. 2000; 275:30873–7. [PubMed: 10906139]

Ding Q, He X, Hsu JM, Xia W, Chen CT, Li LY, Lee DF, Liu JC, Zhong Q, Wang X, Hung MC. Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Mol Cell Biol*. 2007; 27:4006–17. [PubMed: 17387146]

Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*. 2006; 7:606–19. [PubMed: 16847462]

Fernandez-Checa JC, Hirano T, Tsukamoto H, Kaplowitz N. Mitochondrial glutathione depletion in alcoholic liver disease. *Alcohol*. 1993; 10:469–75. [PubMed: 8123202]

French BA, Oliva J, Bardag-Gorce F, Li J, Zhong J, Buslon V, French SW. Mallory-Denk bodies form when EZH2/H3K27me3 fails to methylate DNA in the nuclei of human and mice liver cells. *Exp Mol Pathol*. 2012; 92:318–26. [PubMed: 22465358]

French SW. The Mallory body: structure, composition, and pathogenesis. *Hepatology*. 1981; 1:76–83. [PubMed: 6269976]

French SW. Present understanding of the development of Mallory's body. *Arch Pathol Lab Med*. 1983; 107:445–50. [PubMed: 6192789]

French SW, Bardag-Gorce F, French BA, Li J, Oliva J. The role of innate immunity in the pathogenesis of preneoplasia in drug-induced chronic hepatitis based on a mouse model. *Exp Mol Pathol*. 2011; 91:653–9. [PubMed: 21820428]

French SW. How to prevent alcoholic liver disease. *Exp Mol Pathol*. 2015; 98:304–7. [PubMed: 25758202]

French SW, Liao G, Li J, Vitocruz E, Liu H, Tillman B, French BA, Mendoza AS. What are the mechanisms of regeneration inhibition in alcoholic hepatitis? *Exp Mol Pathol*. 2016; 100:502–5. [PubMed: 27189521]

Gobessi S, Laurenti L, Longo PG, Carsetti L, Berno V, Sica S, Leone G, Efremov DG. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia*. 2009; 23:686–97. [PubMed: 19092849]

Goodman PA, Wood CM, Vassilev A, Mao C, Uckun FM. Spleen tyrosine kinase (Syk) deficiency in childhood pro-B cell acute lymphoblastic leukemia. *Oncogene*. 2001; 20:3969–78. [PubMed: 11494125]

Haybaeck J, Stumptner C, Thueringer A, Kolbe T, Magin TM, Hesse M, Fickert P, Tsybrovskyy O, Muller H, Trauner M, Zatloukal K, Denk H. Genetic background effects of keratin 8 and 18 in a DDC-induced hepatotoxicity and Mallory-Denk body formation mouse model. *Lab Invest*. 2012; 92:857–67. [PubMed: 22449798]

- Hoyer-Hansen M, Jaattela M. AMP-activated protein kinase: a universal regulator of autophagy? *Autophagy*. 2007; 3:381–3. [PubMed: 17457036]
- Hu B, French SW. Mallory body and empty cell in hepatitis C. *Hepatol Res*. 1997a; 8:13–20.
- Hu B, French SW. 2',3'-Dideoxyinosine-induced Mallory bodies in patients with HIV. *Am J Clin Pathol*. 1997b; 108:280–3. [PubMed: 9291455]
- Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett*. 2010; 584:1287–95. [PubMed: 20083114]
- Kachi K, Wong PT, French SW. Molecular structural changes in Mallory body proteins in human and mouse livers: an infrared spectroscopy study. *Exp Mol Pathol*. 1993; 59:197–210. [PubMed: 8137902]
- Kulathu Y, Grothe G, Reth M. Autoinhibition and adapter function of Syk. *Immunol Rev*. 2009; 232:286–99. [PubMed: 19909371]
- Lackner C, Gogg-Kamerer M, Zatloukal K, Stumppner C, Brunt EM, Denk H. Ballooned hepatocytes in steatohepatitis: the value of keratin immunohistochemistry for diagnosis. *J Hepatol*. 2008; 48:821–8. [PubMed: 18329127]
- Leibach WK. Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse. *Ann N Y Acad Sci*. 1975; 252:85–105. [PubMed: 1096716]
- Leseux L, Hamdi SM, Al Saati T, Capilla F, Recher C, Laurent G, Bezombes C. Syk-dependent mTOR activation in follicular lymphoma cells. *Blood*. 2006; 108:4156–62. [PubMed: 16912221]
- Liu H, Gong M, French BA, Li J, Tillman B, French SW. Mallory-Denk Body (MDB) formation modulates Ufm1ylation expression epigenetically in alcoholic hepatitis (AH) and non-alcoholic steatohepatitis (NASH). *Exp Mol Pathol*. 2014a; 97:477–83. [PubMed: 25290169]
- Liu H, Li J, Tillman B, French BA, French SW. Ufm1ylation and FATylation pathways are downregulated in human alcoholic and nonalcoholic steatohepatitis, and mice fed DDC, where Mallory-Denk bodies (MDBs) form. *Exp Mol Pathol*. 2014b; 97:81–8. [PubMed: 24893112]
- Liu H, Gong M, French BA, Liao G, Li J, Tillman B, French SW. Aberrant modulation of the BRCA1 and G1/S cell cycle pathways in alcoholic hepatitis patients with Mallory Denk Bodies revealed by RNA sequencing. *Oncotarget*. 2015; 6:42491–503. [PubMed: 26623723]
- Mallory FB. Giant Cell Sarcoma. *J Med Res*. 1911; 24:463–468 3. [PubMed: 19972006]
- Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di Lisi R, Sandri C, Zhao J, Goldberg AL, Schiaffino S, Sandri M. FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab*. 2007; 6:458–71. [PubMed: 18054315]
- Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007; 129:1261–74. [PubMed: 17604717]
- Masouminia M, Samadzadeh S, Mendoza AS, French BA, Tillman B, French SW. Upregulation of autophagy components in alcoholic hepatitis and nonalcoholic steatohepatitis. *Exp Mol Pathol*. 2016a; 101:81–8. [PubMed: 27432584]
- Masouminia M, Samadzadeh S, Ebaee A, French BA, Tillman B, French SW. Alcoholic steatohepatitis (ASH) causes more UPR-ER stress than non-alcoholic steatohepatitis (NASH). *Exp Mol Pathol*. 2016b; 101:201–206. [PubMed: 27587085]
- Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol*. 2010; 10:387–402. [PubMed: 20467426]
- Nakanuma Y, Ohta G. Is mallory body formation a preneoplastic change? A study of 181 cases of liver bearing hepatocellular carcinoma and 82 cases of cirrhosis. *Cancer*. 1985; 55:2400–4. [PubMed: 2985233]
- Qin S, Minami Y, Hibi M, Kurosaki T, Yamamura H. Syk-dependent and -independent signaling cascades in B cells elicited by osmotic and oxidative stress. *J Biol Chem*. 1997; 272:2098–103. [PubMed: 8999908]
- Sada K, Zhang J, Siraganian RP. SH2 domain-mediated targeting, but not localization, of Syk in the plasma membrane is critical for FcεRI signaling. *Blood*. 2001a; 97:1352–9. [PubMed: 11222380]
- Sada K, Takano T, Yanagi S, Yamamura H. Structure and function of Syk protein-tyrosine kinase. *J Biochem*. 2001b; 130:177–86. [PubMed: 11481033]

- Thakur V, McMullen MR, Pritchard MT, Nagy LE. Regulation of macrophage activation in alcoholic liver disease. *J Gastroenterol Hepatol*. 2007; 22(Suppl 1):S53–6. [PubMed: 17567466]
- Thiele GM, Klassen LW, Tuma DJ. Formation and immunological properties of aldehyde-derived protein adducts following alcohol consumption. *Methods Mol Biol*. 2008; 447:235–57. [PubMed: 18369923]
- Tuma DJ, Casey CA. Dangerous byproducts of alcohol breakdown--focus on adducts. *Alcohol Res Health*. 2003; 27:285–90. [PubMed: 15540799]
- Viitala K, Makkonen K, Israel Y, Lehtimäki T, Jaakkola O, Koivula T, Blake JE, Niemela O. Autoimmune responses against oxidant stress and acetaldehyde-derived epitopes in human alcohol consumers. *Alcohol Clin Exp Res*. 2000; 24:1103–9. [PubMed: 10924016]
- Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*. 2002; 2:489–501. [PubMed: 12094235]
- Wang J, Cornford ME, German J, French SW. Sclerosing hyaline necrosis of the liver in Bloom syndrome. *Arch Pathol Lab Med*. 1999; 123:346–50. [PubMed: 10320150]
- Wang L, Duke L, Zhang PS, Arlinghaus RB, Symmans WF, Sahin A, Mendez R, Dai JL. Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. *Cancer Res*. 2003; 63:4724–30. [PubMed: 12907655]
- Yuan QX, Marceau N, French BA, Fu P, French SW. Mallory body induction in drug-primed mouse liver. *Hepatology*. 1996; 24:603–12. [PubMed: 8781332]
- Zatloukal K, French SW, Stumptner C, Strnad P, Harada M, Toivola DM, Cadrin M, Omary MB. From Mallory to Mallory-Denk bodies: what, how and why? *Exp Cell Res*. 2007; 313:2033–49. [PubMed: 17531973]
- Zhang J, Benavente CA, McEvoy J, Flores-Otero J, Ding L, Chen X, Ulyanov A, Wu G, Wilson M, Wang J, Brennan R, Rusch M, Manning AL, Ma J, Easton J, Shurtleff S, Mullighan C, Pounds Mukatira S, Gupta P, Neale G, Zhao D, Lu C, Fulton RS, Fulton LL, Hong X, Dooling DJ, Ochoa K, Naeve C, Dyson NJ, Mardis ER, Bahrami A, Ellison D, Wilson RK, Downing JR, Dyer MA. A novel retinoblastoma therapy from genomic and epigenetic analyses. *Nature*. 2012; 481:329–34. [PubMed: 22237022]
- Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab*. 2007; 6:472–83. [PubMed: 18054316]

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 p70S6k signaling pathway.

Figure 2.

Figures A, B and C: Liver sections from different patients with AH and control (D) were double stained with antibodies to SYK (green), UB (red) and DAPI (blue). The ballooned hepatocytes stained positive for both SYK in the cytoplasm and UB in the MDBs. Figure E shows the comparison of morphometric measurements of SYK present in the cytoplasm of MDB forming hepatocytes to the surrounding hepatocytes which had not formed MDBs ($P=0.001$). Figure F indicates that SYK has significantly higher expression in AH patients compared to the control samples by quantitative PCR ($P=0.016$).

Figure 3.

Figures A and B: The FFPE liver sections from AH patient and control (figure C) were double stained with antibodies to AKT (green), UB (red) and DAPI (blue). The AH liver sections with MDBs stained with greater intensity for AKT compared to the controls (x....). Figure D: AKT protein measured morphometrically in liver from a patient with alcohol hepatitis and the control (P=0.03). Figure E and F: Western blot analysis of AKT1 in AH and control biopsies. Figure G: The quantitative PCR result indicates that the expression of AKT1 is tended to be increased. Data represent mean values $\pm$ S.E.M.

Figure 4.

Figures A to C: The liver of a patient with alcohol hepatitis and control (Figures D to F) were fixed in formalin and embedded in paraffin (FFPE). They were stained with antibodies to mTOR (green) and UB (red) and viewed under a fluorescent microscope. Figure G: Fluorescent intensity of mTOR protein measured morphometrically in liver from patients with alcohol hepatitis and compared with control ($P=0.02$). Figure H: The quantitative PCR analysis shows that the expression of mTOR is tended to be increased in AH compared to control biopsies.

Figure 5.

Figures A and B: Double staining of FFPE liver tissue from AH patients and control (C) samples with antibodies to PIK3CB (green) and UB(red) indicates significant expression of PIK3CB in the AH patients (X....). Figure D shows the morphometrically measurement of PIK3CB protein in the liver of a patient with alcohol hepatitis and control ($P=0.02$).

Figure 6. Schematic of SYK activation by oxidative stress and calcium mediated events leading to ER stress, induced apoptosis as well as deregulation of autophagocytosis through PI3K/AKT/mTOR pathways

Table 1

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

Species	Symbol	Name	Accession	Size	Sequence
Human	SYK	spleen associated tyrosine kinase	NM_003177	94	Forward: 5'-CAGAAAGCAAATGTCATGCAG-3' Reverse: 5'-ATCTCCATAACCAAGCATCCA-3'
Human	mTOR	mechanistic target of rapamycin	NM_004958	96	Forward: 5'-CGTGGAGAAACATGGATTAGG-3' Reverse: 5'-GTCCACAGACCAAGTGAGGTC-3'
Human	AKT1	v-akt murine thymoma viral oncogene homolog 1	NM_005163	74	Forward: 5'-ATGGAAAGACGTTTTTCTGTC-3' Reverse: 5'-ACCCGCAGGATAGTTTTTCTT-3'
Human	TUBB-1	tubulin beta class I	NM_178014	196	Forward: 5'-ACCAAGGTGCTGAAACACACAT-3' Reverse: 5'-CTTGAAGCTGAGATGGGAAA-3'

Table 2

Up regulated and downregulated genes in p70S6K signaling pathway (Liu et al., 2015).

Gene Symbol	gene name	Fold Change	p-value (ASH vs. Control)	
PLCD3	phospholipase C, delta 3	12.8195	0.035654	AH up regulated vs Control
CD19	CD19 molecule	52.6429	0.027566	AH up regulated vs Control
IL2RG	Interleukin 2 Receptor Subunit Gamma	27.7813	0.0024253	AH up regulated vs Control
SYK	spleen associated tyrosine kinase	4.60465	0.00840433	AH up regulated vs Control
GNAI1	G Protein Subunit Alpha I1	6.4	0.00974514	AH up regulated vs Control
PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide	8.59712	0.0348563	AH up regulated vs Control
SFN	Stratifin	11.5195	0.00801286	AH up regulated vs Control
PLCL1	Phospholipase C Like 1	110	0.0488749	AH up regulated vs Control
ATM	ATM Serine/Threonine Kinase	2.70231	0.0336726	AH up regulated vs Control
MAP2K1	Mitogen-Activated Protein Kinase Kinase 1	-2.33368	0.0145019	AH down regulated vs Control

Table 3

The ingenuity Canonical Pathways that have impacted by SYK and PIK3CB (Liu et al., 2015)

Ingenuity Canonical Pathways	−log(p-value)	Ratio	z-score	Molecules
p70S6K Signaling	1.92E00	8.47E-02	2.333	SYK,PIK3CB
Tec Kinase Signaling	1.89E00	7.69E-02	2.121	PIK3CB
CCR3 Signaling in Eosinophils	1.62E00	7.96E-02		PIK3CB
Prostate Cancer Signaling	1.55E00	8.75E-02		PIK3CB
p53 Signaling	1.55E00	8.16E-02	0.000	PIK3CB
IL-9 Signaling	1.45E00	1.18E-01	2.000	PIK3CB
IL-8 Signaling	1.41E00	6.56E-02	2.309	PIK3CB
UVA-Induced MAPK Signaling	1.38E00	8.05E-02	2.000	PIK3CB
Role of Tissue Factor in Cancer	1.35E00	7.48E-02		PIK3CB
Molecular Mechanisms of Cancer	1.35E00	5.57E-02		PIK3CB
Natural Killer Cell Signaling	1.33E00	7.41E-02		SYK,PIK3CB
G-Protein Coupled Receptor Signaling	1.3E00	5.91E-02		PIK3CB