Experimental and Molecular Pathology 102 (2017) 106–114

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

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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ARTICLE INFO

Article history:
Received 11 January 2017
Accepted 12 January 2017
Available online 13 January 2017

Keywords:
Alcoholic hepatitis
SYK
AKT1
Mallory-Denk bodies

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.

1. 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 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, 1997a,b), 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

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http://dx.doi.org/10.1016/j.yexmp.2017.01.011
0014-4800/

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 preneoplasmic 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.

2. Material and methods

2.1. 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, 2014b). Liver biopsy sections were cut 5 μm 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.

2.2. 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 min. The slides were then submerged in xylene at room temperature for 1 h changing the xylene once after 30 min. The samples were hydrated by washing progressively for 2 min in 100%, 70%, 50% ethanol, and then pure RNase-free water before air-drying the samples on the slides for approximately 10 min. 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.

2.3. 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.

2.4. Quantitative real-time PCR analysis

Synthesis of first-strand cDNAs was performed with the above mentioned total RNA (1 μg), 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 s, followed by 40 cycles of 95 °C for 3 s. 60 °C for 30 s. 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 ΔΔCT method. Reaction of each sample was performed in triplicate.

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′-CAGAAGCAAATGTCATGCAG-3′ Reverse: 5′-ATCTCCATAACCAGCATCCA-3′
Human	mTOR	Mechanistic target of rapamycin	NM_004958	96	Forward: 5′-CGTGGAGAACATGGATTAGG-3′ Reverse: 5′-GTCCACAGACCAGTGAGGTC-3′
Human	AKT1	v-akt murine thymoma viral oncogene homolog 1	NM_005163	74	Forward: 5′-ATGGAAAGACGTTTTTGTGC-3′ Reverse: 5′-ACCCGCAGGATAGTTTTCTT-3′
Human	TUBB-1	Tubulin beta class I	NM_178014	196	Forward: 5′-ACCAGGTGCTGAAAACACAT-3′ Reverse: 5′-CTTGAAGCTGAGATGGGAAA-3′