A role for mammalian target of rapamycin (mTOR) pathway in non alcoholic steatohepatitis related-cirrhosis

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Summary. Non-alcoholic fatty liver disease (NAFLD) encompasses the whole spectrum of steatosis, non-alcoholic steatohepatitis (NASH), and NASH-related cirrhosis (NASH/Cir). Although molecular advances have been made in this field, the pathogenesis of NAFLD is not completely understood. The gene expression profiling associated to NASH/Cir was assessed, in an attempt to better characterize the pathways involved in its etiopathogenesis. Methods: In the first step, we used cDNA microarray to evaluate the gene expression profiles in normal liver (n=3) and NASH/Cir samples (n=3) by GeneSifter™ analysis to identify differentially expressed genes and biological pathways. Second, tissue microarray was used to determine immunohistochemical expression of phosphorylated mTOR and 4E-BP1 in 11 normal liver samples, 10 NASH/Cir samples and in 37 samples of cirrhosis of other etiologies to further explore the involvement of the mTOR pathway evidenced by the gene expression analysis. Results: 138 and 106 genes were, respectively, up and down regulated in NASH/Cir in comparison to normal liver. Among the 9 pathways identified as significantly modulated in NASH/Cir, the participation of the mTOR pathway was confirmed, since expression of cytoplasmic and membrane phospho-mTOR were higher in NASH/Cir in comparison to cirrhosis of other etiologies and to normal liver.

Conclusions: Recent findings have suggested a role for the cellular "nutrient sensor" mTOR in NAFLD and the present study corroborates the participation of this pathway in NASH/Cir. Phospho-mTOR evaluation might be of clinical utility as a potential marker for identification of NASH/Cir in cases mistakenly considered as cryptogenic cirrhosis owing to paucity of clinical data.

Key words: Cirrhosis, Non alcoholic steatohepatitis, Gene expression profile, Microarray analysis, mTOR pathway

Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses the whole spectrum of fatty liver, including non-alcoholic steatosis, steatohepatitis (NASH), and NASH-related cirrhosis (NASH/Cir) in patients without a history of alcohol abuse (McCullough et al., 2002). NASH associated with cirrhosis can decompensate into sub acute liver failure, progress to hepatocellular cancer (HCC) and reoccur post transplantation (Ong et al., 2001; Bugianesi et al., 2002; Caldwell et al., 2002). Although the risk factors for NAFLD are well established and molecular advances have been made in

Abbreviations. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; NASH/Cir: NASH-related cirrhosis; mTOR: mammalian target of rapamycin; 4E-BP1: 4E binding protein 1; TMA: Tissue Microarray

knowing the complex pathogenesis of NAFLD (Sreekumar et al., 2003; Younossi et al., 2005; Chiappini et al., 2006), the natural history of this condition in humans is still uncertain.

The study of patterns of intra hepatic gene expression in normal and diseased liver tissue is a necessary prerequisite to increase the understanding of these processes, particularly in human liver disease as well as seeking to identify molecular markers that will facilitate early diagnosis and accurate staging of the disease.

The analysis of differential gene expression has already been used to identify molecules and pathways associated to cirrhosis caused by chronic viral hepatitis (Shackel et al., 2003; Smith et al., 2003), hemochromatosis, Wilson's disease, alcoholic liver disease, autoimmune hepatitis, primary biliary cirrhosis (Kim et al., 2004) and NASH (Younossi et al., 2005).

The goal of the present study was to assess the role of mTOR and its downstream effector 4EBP-1 in NASH-related cirrhosis in an attempt to better characterize the involvement of this pathway in its etiopathogenesis.

Materials and methods

Tissue collection and RNA isolation

Hepatic tissue was obtained from three patients (two male and one female, mean age 64 years) affected by NASH/Cir during orthotopic liver transplantation at the Department of Gastroenterology, Liver Transplantation, Medical School, University of Sao Paulo. Fragments of liver tissues were fixed in formaldehyde saline (4%) and processed for hematoxylin-eosin (HE) and Masson Trichrome stains. The diagnosis of NASH/Cir was made only after an exhaustive evaluation failed to define other specific etiologies: absence of serologic markers for known liver diseases; lack of evidence of chronic alcohol and potentially hepatotoxic drug ingestion; negative serologic testing for autoimmune hepatitis (antibodies to nuclear/mitochondrial/smooth muscle antigens), for hemochromatosis (ferritin, iron, iron binding capacity), for Wilson's disease (ceruloplasmin concentrations) and for alpha-1 antitrypsin deficiency (α-1 trypsin levels); negative histopathological findings for hemochromatosis, for alpha-1 antitrypsin deficiency and for primary biliary cirrhosis. Histological markers of NAFLD activity (steatosis 0-3, ballooning 0-2 and lobular inflammation 0-3) were assessed according to Histological Scoring System for Nonalcoholic Fatty Liver Disease (NASH Activity Score or NAS), recently published by the Pathology Committee of the NASH Clinical Research Network (Kleiner et al., 2005), which considers a score 4 consistent with NASH. Two of the three patients presented a score 4 in the histopathological evaluation performed on liver sections taken at transplantation and the third patient presented a score 3, although a previous hepatic biopsy performed three

years before transplantation presented a score 6, confirming the histologic diagnostic of NASH.

Normal liver tissue was obtained from donor wedge biopsies taken at transplantation (two male and one female donors, mean age 47 years). Biological triplicate was used to reduce the effect of individual phenotypic differences for each group. This study was approved by the Ethical Committee of Hospital das Clinicas of the University of São Paulo, School of Medicine and in accordance to The Declaration of Helsinki, with informed and free consent being required of each subject or subject's guardian. All liver tissue samples were collected in sterile containers and immediately snap frozen in liquid nitrogen after surgical removal and stored at -80°C until their use.

Total RNA was isolated from frozen liver samples after mechanical disruption and extraction by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guidelines. Further, the precipitated products were purified with RNeasy™ Mini Kit (Qiagen, Hilden, Germany).

The quality of total RNA samples was analyzed by inspection of 18S and 28S rRNA bands following agarose gel electrophoresis. The concentration of RNA samples was quantified by measurement of the optical density using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Microarray analysis

We utilized the CodeLink™ Human Whole Genome Bioarray (GE Healthcare Biosciences, Chalfont St. Giles, UK) with ~57,000 human transcripts represented in a single bioarray.

Target preparation, hybridization, and probe array processing (washing, staining, and scan) were performed according to the protocols in the manufacturer's instructions. Briefly, 5 μg of total RNA was first reverse transcribed to the single-stranded cDNA and subsequent cRNA was synthesized using the CodeLink™ Expression Assay Kit (GE Healthcare Biosciences). The cRNA targets were prepared by in vitro transcription using a single, labeled nucleotide, biotin-11-UTP at a concentration of 1.25 mM. Unlabeled UTP was present at 3.75 mM, while GTP, ATP, and CTP were at 5 mM. The mixture was incubated at 37°C overnight for 14 hours. The labeled cRNA was then purified using RNeasy™ mini kit (Qiagen) and subsequently fragmented in 1x fragmentation buffer (40 mM Tris-acetate pH 7.9, 100 mM KOAc, 31.5 mM MgOAc) at 94°C for 20 minutes.

For hybridization, 10 μg of fragmented cRNA in 260 μl of hybridization solution was added to each bioarray and incubated for 18 hours at 37°C, while shaking at 300 rpm in a shaking incubator. Immediately following hybridization, the bioarrays were washed and stained with Cy5™-streptavidin (GE Healthcare Biosciences) and scanned using a GenePix® 4000B Array Scanner (Axon Instruments, Union City, CA, USA).

Microarray expression analysis

The quality of microarray data was assessed by monitoring a series of quality control parameters as suggested by CodeLink™ Expression Array Software (GE Healthcare Biosciences), including visually inspecting the array images to confirm scanner alignment and filtering out genes with missing spots. The ~57,000 spots' intensities on the microarray image were quantified, where the intensity of each spot was divided by the array's signal median to provide a scaled and comparable number across multiple arrays. The expression values of these genes were normalized individually across all arrays by taking the intensity values in increasing order subtracted by 0.5 and divided by the size of the sample (Q-value).

Expression ratio was calculated by the difference between the averages of Q-transformed values from 3 NASH/Cir and 3 normal liver arrays, multiplied by 10. Unpaired Student's t-test was used for two-group statistical comparisons. Differentially expressed genes were selected according to 3 basic criteria: presence of at least 40% of "G" (good) flag in analyzed arrays, statistically significant ratio at probability levels of $p < 0.05$ and differential expression either up- or down-regulated by at least 2-fold in the average to normal livers compared to NASH/Cir data.

Gene ontology analysis using gene ontology

Microarray gene expression data were analyzed by GeneSifter™ program (http://www.genesifter.net/web/). Gene ontology routine included in the program was chosen for further interpretation of the data regarding molecular function related genes according to Gene Ontology (GO) Consortium categories (http://www.geneontology.org/GO.doc.html) (Ashburner et al., 2000).

All the pathways in the GeneSifter™ database were examined to determine whether a significant number of altered genes in GO term were affected. We examined z-scores that express the frequency of genes fulfilling an increase ≥2 in each GO term and compared with null hypothesis expected frequency for that GO term based on the total number of genes examined on the array. The z-score was derived by dividing the difference between the observed number of genes meeting the criterion in a specific GO term and the expected number of genes based on the total number of genes in the array meeting the criterion. This value was then divided by the standard deviation of the observed number of genes under a hypergeometric distribution.

Positive z-scores indicate GO terms with a greater number of genes meeting the criterion than is expected by chance. Negative z-scores indicate GO terms with fewer genes meeting the criterion than expected by chance. A z-score near zero indicates that the number of genes meeting the criterion approximates the expected number. A z-score of greater than 2 is considered a statistically significant association (approximately equivalent to a $p \leq 0.05$) between the differentially regulated genes and their corresponding GO terms (Ashburner et al., 2000).

Correction for multiple testing was then performed using the method of Reiner et al., 2003 to derive a false discovery rate estimate from raw p-values. A false discovery rate of 5% was also set as a cutoff for statistical significance.

Tissue microarray (TMA)

From 2003 to 2007, hepatic cirrhosis cases from different etiologies: NASH/Cir (n=10), HCV-related cirrhosis (n=9), HBV-related cirrhosis (n=9), auto-immune hepatitis (n=9), primary biliary cirrhosis (n=3), ethanol cirrhosis (n=1), Wilson's disease (n=1), α1-anti-tripsyn deficiency (n=2), primary sclerosing cholangitis (n=2), and cryptogenic cirrhosis (n=1) were collected from Anatomic Pathology Division, Hospital das Clinicas, University of Sao Paulo and spotted on a TMA. The diagnosis of NASH/Cir was made as described above. Eleven normal liver samples came from a second TMA.

In brief, hematoxylin and eosin stained slides from the donor cases were reviewed to identify viable, morphologically representative areas of the specimen, and then the areas were marked (2 spots by cases). After transferring of the marks to respective donor paraffin blocks, a TMA block was constructed by punching the spotted areas (1.0 mm punch) and mounting them onto a recipient paraffin block at 0.3 mm intervals between the cores, in a grid system where each core has a coordinate reference (x-axis, y-axis, in a final grid of 9 lines by 10 columns) using a precision microarray instrument (Beecher Instruments, USA). The TMA block was cut at 3 μm interval consecutive sections (Leica Instruments, Germany) (Kononen et al., 1998).

Immunohistochemical protocol

Three μm sections of the TMAs were deparaffinized in xylene for 10 min followed by hydration in serial ethanol dilutions and distilled water. Antigen retrieval was performed by boiling slides in 10 mM sodium citrate pH 6.0 for 40 min in a steamer (Shi et al., 1991). Endogenous peroxidase activity was blocked for 30 min in 6% hydrogen peroxide diluted in methanol. Subsequent incubation with CASBlock™ (Invitrogen/Zymed, USA) was carried out for 10 min at 37°C. After blotting the excess of CASBlock™, slides were incubated with the primary antibodies (anti-phospho 4E-BP1(Thr37/46), rabbit monoclonal, clone 236B4, 1:200, and/or anti-phospho mTOR (Ser2448), rabbit monoclonal, clone 49F9, 1:50 (Cell Signaling Technology Inc, Danvers, USA) for 30 min at 37°C followed by overnight incubation at 4°C. The next day, incubation with a short polymer amplification system (Novolink™, Vision Biosystems, Australia) was applied for 30 min at 37°C. The peroxidase reactivity was

developed by 60 mg 3,3'-diaminobenzidine in PBS buffer (pH 7.4) for 5 min at 37°C. Between all steps, slides were washed in phosphate-buffered saline (pH 7.4). Finally, slides were counterstained with Harris's haematoxylin for 1 min, dehydrated, and mounted in Entellan (Merck, USA). Positive control tissue was run in parallel with another tissue microarray with a varied group of gastrointestinal carcinomas for both proteins. Negative control was obtained by omitting the primary antibody.

Evaluation and scoring

Immunohistochemical staining was quantified conjointly by 2 observers (ICS and MSK) blinded to clinical data using a scoring system that incorporates both staining intensity and percentage of positive hepatocyte cells. For each TMA spot, the intensity of each immunostained protein (0, absent; 1, weak; 2, medium; and 3, strong) was multiplied by the percentage of positive staining hepatocyte cells (from 0 to 100%,

with classes of 10% increment), resulting in a score ranging from 0-300 (Herberger et al., 2007). To represent each case, the mean of the 2 spots of the case presented on the TMA was calculated. Only spots with at least 50% of interest tissue were considered in the computation. Two different cellular compartments were evaluated: cytoplasm and plasmatic membrane, using the same scoring system.

Statistical analysis

Statistical analysis was carried out using GraphPad PRISM software version 2.0 (Kruskal-Wallis and Dunn's multiple comparison tests). Non-parametric two-tailed p-values <0.05 were considered statistically significant.
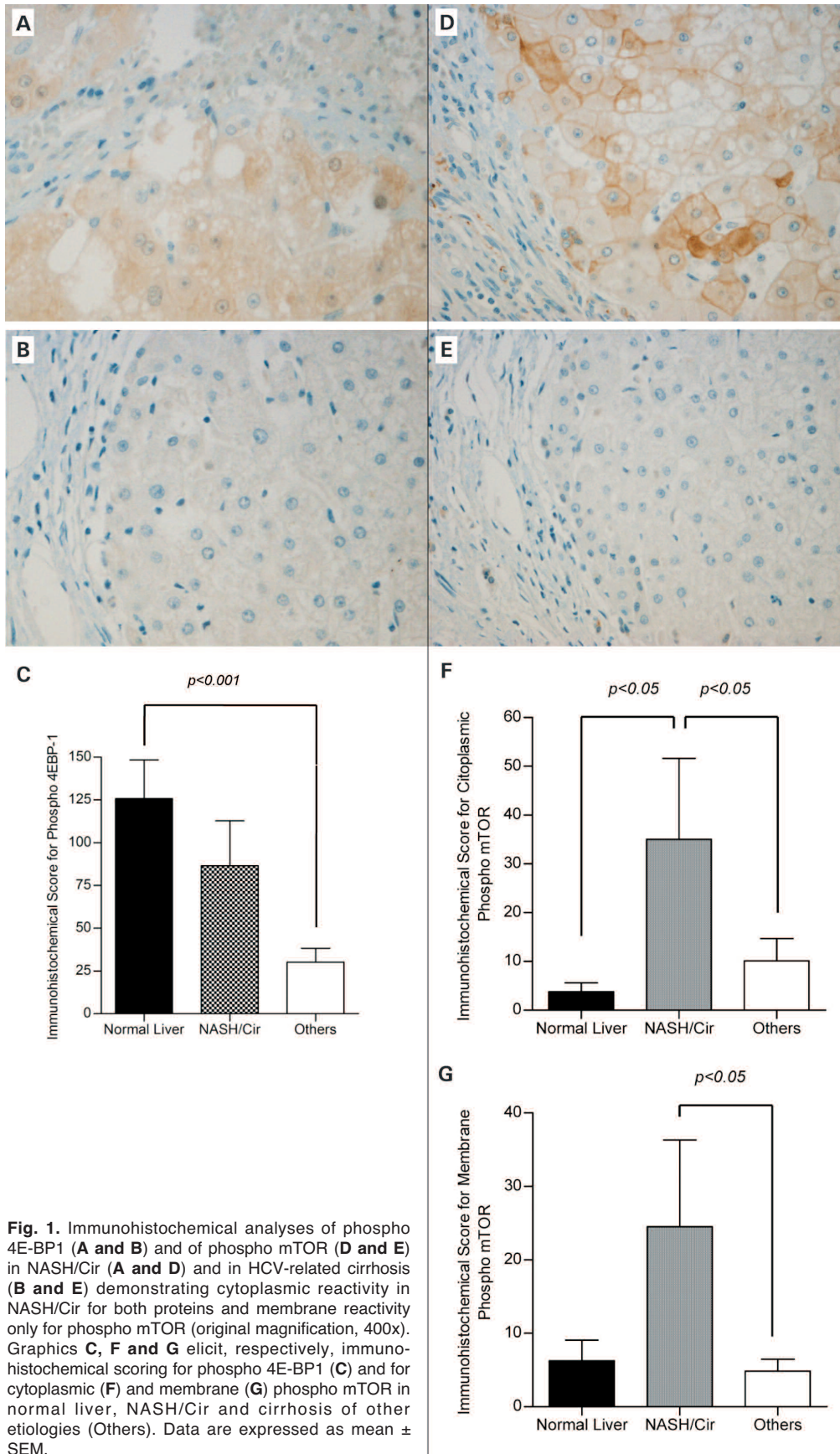
Results

Genomic expression

Using filtering criteria of at least 2.0-fold-change in

Table 1. The down regulated biological pathways and representative genes in NASH-related cirrhosis.

UNIGENE	GENE SYMBOL	PATHWAY z-score	GENE NAME	NASH/Cir Up/Down
Biosynthesis of steroids		9.11		
Hs.503134	DHCR7		7-dehydrocholesterol reductase	down
Hs.632801	EBP		Emopamil binding protein (sterol isomerase)	down
Hs.643476	FDFT1		Farnesyl-diphosphate farnesyltransferase 1	down
Hs.283652	IDI1		Isopentenyl-diphosphate delta isomerase	down
Hs.130607	MVK		Mevalonate kinase (mevalonic aciduria)	down
Hs.71465	SQLE		Squalene epoxidase	down
Hs.287749	SC5DL		Sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like	down
Phosphatidylinositol signaling system		3.09		
Hs.235116	GRK6		G protein-coupled receptor kinase 6	down
Hs.431173	PLCB1		Phospholipase C, beta 1 (phosphoinositide-specific)	up
Hs.580527	INPP4A		Inositol polyphosphate-4-phosphatase, type I	down
Hs.523360	INPP5A		Inositol polyphosphate-5-phosphatase	down
Hs.397465	HIPK2		Homeodomain interacting protein kinase 2	down
Hs.497487	PIK3C2B		Phosphoinositide-3-kinase, class 2, beta polypeptide	down
Hs.371344	PIK3R2		Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	down
Hs.498892	EEF2K		Eukaryotic elongation factor-2 kinase	down
Hs.534371	PIP5K1B		Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	down
Hs.32942	PIK3CG		Phosphoinositide-3-kinase, catalytic, gamma polypeptide	up
Hs.472027	CDS2		CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2	up
Pyrimidine metabolism		2.68		
Hs.473087	CTPS		Cytidine 5'-triphosphate synthetase	down
Hs.599355	DTYMK		Deoxythymidylate kinase (thymidylate kinase)	down
Hs.567352	TXNRD1		Thioredoxin reductase 1	down
Hs.458360	UCK2		Uridine-cytidine kinase 2	down
Hs.591457	POLR3C		Polymerase (RNA) III (DNA directed) polypeptide C	down
Hs.515122	TK1		Thymidine kinase 1	down
Hs.643610	POLR3H		Polymerase (RNA) III (DNA directed) polypeptide H	down
mTOR signaling pathway		2.17		
Hs.411641	EIF4EBP1		Eukaryotic translation initiation factor 4E binding protein 1	down
Hs.431850	MAPK1		Mitogen-activated protein kinase 1	down
Hs.371344	PIK3R2		Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	down
Hs.32942	PIK3CG		Phosphoinositide-3-kinase, catalytic, gamma polypeptide	up
Hs.78781	VEGFB		Vascular endothelial growth factor B	down

Up or down regulation are defined as gene expression in NASH/Cir compared with normal liver tissue.

Fig. 1. Immunohistochemical analyses of phospho 4E-BP1 (**A and B**) and of phospho mTOR (**D and E**) in NASH/Cir (**A and D**) and in HCV-related cirrhosis (**B and E**) demonstrating cytoplasmic reactivity in NASH/Cir for both proteins and membrane reactivity only for phospho mTOR (original magnification, 400x). Graphics **C, F and G** elicit, respectively, immuno-histochemical scoring for phospho 4E-BP1 (**C**) and for cytoplasmic (**F**) and membrane (**G**) phospho mTOR in normal liver, NASH/Cir and cirrhosis of other etiologies (Others). Data are expressed as mean ± SEM.

expression and a t-test where p<0.05, 244 differentially expressed genes were identified, 138 of these genes were up regulated in the NASH/Cir group while 106 genes were down regulated in comparison to the normal liver group.

Gene ontology (GO) analysis by GeneSifter[TM]

Table 2. The up regulated biological pathways and representative genes in NASH-related cirrhosis.

UNIGENE	GENE SYMBOL	PATHWAY z-score	GENE NAME	NASH/Cir Up/Down
T cell receptor signaling pathway		2,94		
Hs.466907	CBLC		Cas-Br-M (murine) ecotropic retroviral transforming sequence c	down
Hs.591629	CD28		CD28 antigen (Tp44)	up
Hs.558348	ITK		IL2-inducible T-cell kinase	up
Hs.193717	IL10		Interleukin 10	down
Hs.390616	PAK3		p21 (CDKN1A)-activated kinase 3	up
Hs.497487	PIK3C2B		Phosphoinositide-3-kinase, class 2, beta polypeptide	down
Hs.371344	PIK3R2		Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	down
Hs.591127	RASGRP1		RAS guanyl releasing protein 1 (calcium and DAG-regulated)	up
Hs.32942	PIK3CG		Phosphoinositide-3-kinase, catalytic, gamma polypeptide	up
Hs.198998	CHUK		Conserved helix-loop-helix ubiquitous kinase	down
ECM-receptor interaction		2,51		
Hs.213861	LAMA4		Laminin, alpha 4	up
Hs.17441	COL4A1		Collagen, type IV, alpha 1	up
Hs.508010	FNDC3A		Fibronectin type III domain containing 3	down
Hs.72550	HMMR		Hyaluronan-mediated motility receptor (RHAMM)	down
Hs.520525	FNDC1		Fibronectin type III domain containing 1	up
Hs.591484	LAMC2		Laminin, gamma 2	up
Cytokine-cytokine receptor interaction		2,39		
Hs.73853	BMP2		Bone morphogenetic protein 2	up
Hs.546294	CCL17		Chemokine (C-C motif) ligand 17	up
Hs.514821	CCL5		Chemokine (C-C motif) ligand 5	up
Hs.34526	CXCR6		Chemokine (C-X-C motif) receptor 6	up
Hs.507590	FLT3		Fms-related tyrosine kinase 3	up
Hs.193717	IL10		Interleukin 10	down
Hs.591742	IL7R		Interleukin 7 receptor	up
Hs.624	IL8		Interleukin 8	up
Hs.846	IL8RB		Interleukin 8 receptor, beta	down
Hs.78781	VEGFB		Vascular endothelial growth factor B	down
Hs.488293	EGFR		Epidermal growth factor receptor	up
Regulation of actin cytoskeleton		2,37		
Hs.525572	BDKRB1		Bradykinin receptor B1	up
Hs.525572	BDKRB2		Bradykinin receptor B2	up
Hs.479747	BCAR1		Breast cancer anti-estrogen resistance 1	down
Hs.287370	FGF23		Fibroblast growth factor 23	up
Hs.522373	GSN		Gelsolin (amyloidosis, Finnish type)	up
Hs.199763	SSH1		Slingshot homolog 1 (Drosophila)	up
Hs.410092	F2		Coagulation factor II (thrombin)	down
Hs.431850	MAPK1		Mitogen-activated protein kinase 1	down
Hs.390616	PAK3		p21 (CDKN1A)-activated kinase 3	up
Hs.497487	PIK3C2B		Phosphoinositide-3-kinase, class 2, beta polypeptide	down
Hs.371344	PIK3R2		Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	down
Hs.517228	TIAM1		T-cell lymphoma invasion and metastasis 1	down
Hs.534371	PIP5K1B		Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	down
Hs.32942	PIK3CG		Phosphoinositide-3-kinase, catalytic, gamma polypeptide	up
Hs.488293	EGFR		Epidermal growth factor receptor	up
Complement and coagulation cascades		2,1		
Hs.525572	BDKRB1		Bradykinin receptor B1	up
Hs.525572	BDKRB2		Bradykinin receptor B2	up
Hs.1321	F12		Coagulation factor XII (Hageman factor)	up
Hs.481992	C6		Complement component 6	down
Hs.93210	C8A		Complement component 8, alpha polypeptide	down
Hs.410092	F2		Coagulation factor II (thrombin)	down
Hs.516578	TFPI		Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	up

Up or down regulation are defined as gene expression in NASH/Cir compared with normal liver tissue

software identified 9 statistically significant pathways containing 68 non-redundant genes differentially expressed. Biosynthesis of steroids, Phosphatidylinositol signaling system, Pyrimidine metabolism and mTOR signaling pathway were identified as pathways down regulated in NASH/Cir in comparison to normal liver, with z-scores of 9.11, 3.09, 2.68 and 2.17, respectively. T cell receptor signaling pathway, ECM-receptor interaction, Cytokine-cytokine receptor interaction, Regulation of actin cytoskeleton and Complement and coagulation cascades pathways were identified as pathways up regulated in NASH/Cir in comparison to normal liver, with z-scores of 2.94, 2.51, 2.39, 2.37 and 2.1 respectively (Tables 1, 2).

Immunohistochemical analysis

Cytoplasmic immunoreactivity for phospho-mTOR was demonstrated in 8 out of 10 (80%) NASH/Cir, in 14 out of 37 (38%) cirrhosis of other etiologies and in 4 out of 11 (36%) normal liver samples, while membrane immunostaining was found in 8 out of 10 (80%) NASH/Cir, in 12 out of 37 (32%) cirrhosis of other etiologies and in 5 out of 11 (45%) normal liver samples. The evaluation of the phosphorylated form of 4E-BP1, a downstream effector of mTOR (whose corresponding gene was identified as down regulated in the microarray analysis in comparison to normal liver) demonstrated its expression in 7 out of 10 (70%) NASH/Cir, in 17 out of 37 (46%) of the cirrhosis of other etiologies and in 10 out of 11 (91%) normal liver samples.

Data of semi-quantitative analysis of immunohisto-chemical stainings are elicited in Figure 1. Expression of cytoplasmic phospho mTOR was significantly higher in NASH/Cir compared with cirrhosis of other etiologies and to normal liver (Graphic F), while membrane reactivity for phospho mTOR was significantly higher only in NASH/Cir in comparison to cirrhosis of other etiologies (Graphic G). Expression of phospho 4E-BP1 was higher in normal liver and in NASH/Cir compared to cirrhosis of other etiologies, although, statistical significance was not reached between NASH/Cir and cirrhosis of other etiologies (Graphic C).

Discussion

The present study identified pathways potentially involved in the etiopathogenesis of NASH-related cirrhosis by the use of human whole-genome microarray hybridization, an approach that allows the obtention of global transcriptional changes to investigate mechanisms underlying the development of diseases. The pathway analysis herein used resulted in the identification of gene networks such as, Pyrimidine metabolism, Biosynthesis of steroids, Phosphatidylinositol signaling system and mTOR signaling pathway, down regulated in NASH-related cirrhosis in comparison to normal liver, and T cell receptor signaling pathway, ECM-receptor interaction, Cytokine-cytokine receptor interaction, Regulation of actin cytoskeleton and Complement and coagulation cascades, up regulated in NASH-related cirrhosis.

Augmented utilization and catabolism of purine and pyrimidine nucleotides has been described after partial hepatectomy or hepatic lesion, indicating the requirement of substrates for salvage nucleotide synthesis during hepatic regeneration (Hashimoto et al., 1998). The down regulation of this pathway in NASH-related cirrhosis is in agreement with previous findings demonstrating decreased DNA synthesis in cirrhotic patients and animals submitted to hepatic surgery (Leong et al., 2006) and probably represent an alteration related to end-stage liver disease, not necessarily associated with NASH. On the other hand, down regulation of several genes belonging to the Steroid biosynthesis pathway, including four of the seven genes herewith identified (DHCR, FDFT1, IDI1 and MV) could be important in the progression of NAFLD. These genes were previously reported as down regulated in rat hepatocytes exposed to arginine deprivation and the authors interpreted this as reflecting an endoplasmatic reticulum (ER) stress (Ozcan et al., 2004). Additionally, it has recently been found that chronic ER stress was present in liver tissue of dietary (high fat diet-induced) and genetic (ob/ob) models of mouse obesity and was able to interfere with insulin receptor signaling and inhibit insulin action in liver cells. Thus, ER stress is being regarded as a molecular link between obesity, insulin resistance, and type 2 Diabetes (Marshall, 2006), which make it plausible to consider its participation in NAFLD development and progression.

Considering the central role of activated hepatic stellate cells in extracellular matrix (ECM) production and hepatic fibrogenesis (Friedman, 2003; Parsons et al., 2007), the identification of ECM-receptor interaction as a disturbed pathway, with up regulation of genes which code for laminin and collagen Type IV in NASH-related cirrhosis is expected and likely to reflect the end-stage liver disease, irrespective of etiology. The same is probably true for the T-Cell receptor signaling pathway, Cytokine-cytokine receptor interaction, Regulation of actin cytoskeleton and Complement and coagulation cascades pathways, whose activation may have a close relationship with the inflammatory response of injured liver (Pinzani and Marra, 2001; Boisvert et al., 2003; Assy et al., 2005).

The mTOR pathway is recognized as an important mediator of a range of anabolic processes, functioning as a nutrient-sensing transductional pathway and interacting with other sensors that detect changes in nutrient availability, such as insulin and AMPK signaling pathways (Khamzina et al., 2005; Yang and Guan, 2007). Recent reports of the involvement of the mTOR pathway in the development of hepatic steatosis and insulin resistance following a high fat diet suggested a role for this pathway in NAFLD (Korsheninnikova et

al., 2005; Calvert et al., 2007) and motivated us to further investigate the gene expression findings, comparing the protein expression of the active phosphorylated forms of mTOR and 4E-BP1 between NASH-related cirrhosis and cirrhosis of other etiologies, in an attempt to find markers specific for NASH-related cirrhosis. The expression of phosphorylated mTOR was significantly higher in NASH-related cirrhosis in comparison to cirrhosis of other causes, suggesting the involvement of mTOR pathway until the final stages of NAFLD. Although these findings might seem contradictory to the one obtained in the microarray analysis, which pointed to a down regulation of the mTOR pathway in NAFLD, it is theoretically possible that the observed down regulation of the genes belonging to the mTOR pathway constitutes a compensatory cellular response to increased pathway activation.

Given the etiopathogenesis of NAFLD, closely related to nutrient overload, it seems feasible to propose that the mTOR pathway may participate in the pathogenesis of NAFLD until end-stage liver disease and, going further, that deregulated mTOR signaling is a more generalized event, as suggested by the findings of Calvert et al. (2007), who identified members of the AKT/mTOR pathway as being differentially phosphorylated in omental adipose tissue from patients with non-progressive form of NAFLD and those with NASH. These results deserve further investigation since characterization of the role of mTOR in NAFLD progression may open new possibilities of therapeutic intervention. An interesting aspect that also needs additional clarification is the absence of differences in the expression of phospho 4E-BP1, a downstream effector of mTOR, between NASH/Cir and normal liver and cirrhosis of other etiologies. One possible explanation for this dissociation is that a reduction of cellular ATP levels inhibits mTOR-dependent phosphorylation of 4E-BP1 (Fingar and Blenis, 2004) and a condition of energy insufficiency could be present in human cirrhotic livers, as already demonstrated in animal models of cirrhosis, where a decreased ratio of ATP/ADP was detected (Harvey et al., 2000). In favour of this hypothesis is the finding of a significantly lower expression of phospho 4E-BP1 when cirrhotic livers of all etiologies were grouped and compared to normal livers (data not shown).

Conclusions

In summary, we propose that mTOR signaling may be specific for the cirrhosis of this etiology, given their recently described association with impaired insulin receptor signaling (Khamzina et al., 2005; Korsheninnikova et al., 2005; Marshall et al., 2006). Our results corroborate recent findings that mTOR pathway participates in NAFLD pathogenesis and may provide potential markers for identification of NASH-related

cirrhosis in cases mistakenly considered as cryptogenic cirrhosis owing to paucity of clinical data.

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