Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Liver specific deletion of CYLDexon7/8 induces severe biliary damage, fibrosis and increases hepatocarcinogenesis in mice

Toni Urbanik[1], Regina Johanna Boger[1], Thomas Longerich[2], Katharina Becker[1], Karl Roland Ehrenberg[1], Nadine Hövelmeyer[3], Matthias Hahn[3], Marcus Schuchmann[4], Dirk Jäger[1], Ari Waisman[3], Marcus Alexander Wörns[4,†], Henning Schulze-Bergkamen[1,*,†]

[1]*National Center for Tumor Diseases, Department of Medical Oncology, University Clinic of Heidelberg, Heidelberg, Germany;* [2]*Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany;* [3]*Institute for Molecular Medicine, Johannes Gutenberg-University Mainz, Mainz, Germany;* [4]*First Department of Medicine, Johannes Gutenberg-University Mainz, Mainz, Germany*

See Editorial, pages 937–939

Background & Aims: *CYLD* is a tumor suppressor gene that is mutated in familial cylindromatosis, an autosomal dominant predisposition to tumors of skin appendages. Reduced CYLD expression has been observed in other tumor entities, including hepatocellular carcinoma. In the present study, we analyzed the role of CYLD in liver homeostasis and hepatocarcinogenesis *in vivo*.

Methods: Mice with liver-specific deletion of CYLDexon7/8 (*CYLD*^{FF}*xAlbCre*) were generated. Liver tissues were histologically analyzed and oval cell activation was investigated. Hepatocarcinogenesis was induced by diethylnitrosamine/phenobarbital (DEN/PB). Microarray expression profiling of livers was performed in untreated as well as DEN/PB-treated mice. NF-κB signaling was assessed by ELISA, quantitative real-time PCR, and Western blotting.

Results: *CYLD*^{FF}*xAlbCre* hepatocytes and cholangiocytes did not express full-length CYLD (FL-CYLD) protein but showed increased expression of the naturally occurring short-CYLD splice variant (s-CYLD). *CYLD*^{FF}*xAlbCre* mice exhibited a prominent biliary phenotype with ductular reaction and biliary-type fibrosis. In addition, *CYLD*^{FF}*xAlbCre* mice showed a significantly increased sensitivity towards DEN/PB-induced hepatocarcinogenesis. Moreover, we could observe the development of cholangiocellular carcinoma, in line with enhanced oval cell activity. NF-κB-signaling was increased in livers of *CYLD*^{FF}*xAlbCre* mice and likely contributed to the inflammatory and fibrotic response.

Conclusions: The deletion of exon7/8 of the *CYLD* gene activates oval cells, leads to a biliary phenotype, and increases the susceptibility towards carcinogenesis in the liver. Thus, our study presents a novel model of biliary damage and liver fibrosis, followed by cancer development.

Keywords: Hepatocellular carcinoma; Cholangiocellular carcinoma; DEN; NF-κB; TNF-α; Oval cells; Fibrosis.

Received 11 January 2012; received in revised form 4 June 2012; accepted 14 June 2012; available online 21 June 2012

☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2012.07.039.

* Corresponding author. Address: National Center for Tumor Diseases, Department of Medical Oncology, University Clinic of Heidelberg, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany. Tel.: +49 06221 56 0; fax: +49 06221 56 33966.

E-mail address: henning.schulze@med.uni-heidelberg.de (H. Schulze-Bergkamen).

† These authors share last authorship.

Abbreviations: NF-κB, Nuclear factor kappa B; IKK, inhibitor of NF-κB kinase complex; NEMO/IKKγ, NF-κB essential modulator; IκB, inhibitor of NF-κB; BCL-3, B-cell lymphoma-leukemia-3; RIP, Receptor interacting protein; PMH, primary murine hepatocytes; PMC, primary murine cholangiocytes; DEN, diethylnitrosamine; PB, phenobarbital; TAK1, TGF-beta activated kinase 1; IL-1/6, Interleukin 1/6; MCP-1, monocyte chemoattractant protein-1; TRAF2/3/6, Tumor necrosis factor receptor-associated factor 2/3/6; gadd45ß, Growth arrest and DNA damage 45beta; TGF-β, transforming growth factor-beta; K-7/19, Cytokeratin-7/19; HPRT, Hypoxanthine-phosphoribosyltransferase; NIK, NF-κB inducing kinase; TLR-2, Toll like receptor-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Mdr2, Multidrug resistance 2.

Introduction

Ubiquitination is a fundamental post-translational modification, regulating protein expression, localization, and activity. The functional outcome of ubiquitination processes is determined by the linkage type of single or poly-ubiquitin chains to the targeted substrate. Protein degradation is a well-known event as a result of lysine-48 (K-48) linkage of poly-ubiquitin chains [1]. New functional aspects of ubiquitination were determined by analysis of Lys-63-(K-63-) linked poly-ubiquitin chains, which lead to promotion of signaling processes such as protein kinase activation or protein–protein interactions. Ubiquitination is a reversible process, providing an additional cellular tool to control signaling events [2].

The tumor suppressor cylindromatosis gene (*CYLD*) was identified as a gene mutated in familial cylindromatosis, which is an autosomal-dominant predisposition to tumors of the skin appendages. The product of the *CYLD* gene contains a ubiquitin C-terminal hydrolase domain allowing it to remove K-63-linked poly-ubiquitin chains from distinct proteins involved in the NF-κB signaling pathway [3]. A central step in NF-κB induction is activation of the IKK complex [3]. *In vitro* studies have shown that CYLD binds to NEMO, the regulatory subunit of the IKK complex. CYLD removes K-63-linked ubiquitin chains from NEMO, thereby inhibiting proteasomal degradation of the NF-κB inhibitors (IκBs) and nuclear translocation of the NF-κB subunits. It has also been shown that CYLD deubiquitinates the IκB-α homologue BCL-3. In contrast to the NF-κB inhibitory function of IκB-α, BCL-3 acts as an NF-κB co-activator by forming transcriptionally active heterodimers with the NF-κB subunits p50 and p52 [4]. CYLD also targets other proteins involved in the NF-κB pathway including RIP, TAK1, TRAF2, and TRAF6 [3]. *CYLD* knockout mice do not spontaneously develop tumors, but they are more sensitive to chemically-induced skin and colon tumorigenesis [4,5].

Recently, we identified a naturally occurring short splice variant of CYLD (short (s)-CYLD) in $CYLD^{ex7/8}$ mice. The s-CYLD isoform lacks the binding sites for TRAF2 and NEMO, but is still capable of deubiquitinating BCL-3 [6].

CYLD expression was found to be downregulated in hepatocellular carcinoma (HCC) [7,8]. Knockdown of CYLD led to an increase of NF-κB activity in HCC cells *in vitro*, which was accompanied by a reduced sensitivity of HCC cells towards chemotherapy- and TNF-α-mediated apoptosis [8]. However, the current knowledge about the contribution of *CYLD* to liver homeostasis and HCC development is limited.

Cancer

Materials and methods

Generation and genotyping of the liver specific $CYLD^{ex7/8}$ mutant mice ($CYLD^{FF}$xAlbCre)

The generation of $CYLD^{ex7/8}$ mutant mice, harboring two loxP-sites ($CYLD^{FF}$), had been previously described [6]. To generate liver-specific $CYLD^{ex7/8}$ mutant mice, $CYLD^{FF}$ mice were crossed with mice expressing the Cre recombinase (both C57BL/6 background) under control of an albumin promoter. This leads to hepatocyte specific excision of exon 7 and, subsequently, to alternative splicing from exon 6 to exon 9 [6]. $CYLD^{FF}$*xAlbCre* offspring were compared to their control littermates with the genotype $CYLD^{FF}$ (referred to as WT). Animals were bred at the animal facility of the University of Mainz. All experiments were done in accordance with the governmental and institutional guidelines and were performed under written approval of the state animal care commission. PCR-based genotyping was performed using specific primers listed in Supplementary Materials and methods (Supplementary methods Table 1). All experiments were performed with male mice.

Quantitative real-time polymerase chain reaction (q-RT PCR)

Isolation of total RNA and cDNA synthesis were performed as previously described [9]. Specific FL- and s-CYLD mRNA transcripts were quantified using LightCycler® FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) and primers listed in Supplementary Materials and methods, Table 2. Q-RT PCR of *collagen I*, *IL-1*, *IL-6*, *TNF-α*, *MCP-1*, *TGF-β1/2*, *K-7*, *K-19*, *survivin*, *gadd45β*, *BCL-3*, and *GAPDH* was performed using primer kits (Qiagen, Hilden, Germany). mRNA expression was normalized to the expression of the housekeeping gene.

Alanine aminotransferase levels

About 100 μl of blood was collected from the tail vein. Alanine aminotransferase (ALT) was measured at the Institute of Clinical and Laboratory Medicine at the University Hospital Mainz according to standard procedures.

Isolation, culturing, and treatment of primary murine hepatocytes

Hepatocytes were isolated and cultured as previously described [9]. After 24 h, cells were incubated with TNF-α (Biomol, Hamburg, Germany) or SN-50 (Enzo-Life-Science, Lörrach, Germany).

Isolation of primary murine cholangiocytes

See Supplementary Materials and methods.

Induction of carcinogenesis

To induce liver carcinogenesis, a single diethylnitrosamine (DEN) (Sigma–Aldrich, Munich, Germany) i.p. injection (0.05 mg per mouse) was performed at day 7 *post partum*. Promotion of carcinogenesis was achieved by continuously adding phenobarbital (PB) (0.05% w/v) to the drinking water from the age of 3 weeks.

Analysis of livers and immunohistochemistry

Livers were assessed visually and the amount of appearing tumor nodules at the liver surface was counted. Additionally, tumor size (diameter) was measured. To investigate liver architecture and tumor histology, 3-μm thick sections were made from formalin-fixed paraffin-embedded liver tissues and stained with hematoxylin and eosin (H&E). Modified Gomori staining was used to assess fibrotic remodeling and architectural distortion. For detection of active caspase 3, CD3, CD68, and Ki67 positive cells, shock frozen liver tissues were sectioned (5 μm) and further processed using the NovoLink™ Min Polymer Detection System (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. Primary antibodies are listed in Supplementary Materials and methods.

Microarray analysis

See Supplementary Materials and methods.

Western blotting

Tissue lysis, protein extraction, and preparation of nuclear and cytosolic extracts were performed as previously described [8,9]. SDS–PAGE and Western blotting were performed according to standard procedures. Immunodetection was performed using primary antibodies listed in Supplementary Materials and methods.

NF-κB activity ELISA

To quantify NF-κB transcription factor activation, the TransAM® NF-κB Family Transcription Factor Assay Kit (Active Motiv, La Hulpe, Belgium) was used according to the manufacturer's instructions. The assay is based on immobilized oligonucleotides containing NF-κB consensus sites. For each well, 3 μg nuclear cell extract was used.

Statistical analysis

See Supplementary Materials and methods.

Results

Liver specific CYLDex7/8 mutant mice ($CYLD^{FF}$xAlbCre)

The $CYLD^{FF}$*xAlbCre* genotype was confirmed by PCR (Fig. 1A). As shown by Western blot analysis, FL-CYLD expression was not detectable in livers and isolated primary murine hepatocytes (PMH) of 3-month-old $CYLD^{FF}$*xAlbCre* mice. In accordance with our previous findings in $CYLD^{ex7/8}$ knockout B cells [6], $CYLD^{FF}$*xAlbCre* livers and PMH showed an increased protein expression of s-CYLD (Fig. 1B, upper panel).