Table 2 Downregulated miRNAs in hepatocarcinogenesis

miRNA	Expression levels	Targets	Main tested samples	References
let-7a	Downregulated	STAT3	Cultured cells	[94]
let-7c	Downregulated		Human tissues, cultured cells	[95]
let-7g	Downregulated	COL12A	Cultured cells, human tissues	[96]
miR-7	Downregulated	PIK3CD	Cultured cells, human tissues	[97]
miR-10a	Downregulated	EphA4	Cultured cells	[98]
miR-10b	Downregulated		Human tissues	[99]
miR-15a/16	Downregulated		Cultured cells	[100]
miR-21	Downregulated		Human tissues	[82]
miR-26a	Downregulated	IL-6	Human tissues, xenograft model	[101]
	Downregulated	CyclinD2, E2	Cultured cells, mouse model	[102]
miR-29	Downregulated	Bcl2, Mcl1	Human tissues, cultured cells	[103]
miR-29b	Downregulated	MMP-2	Human tissues, cultured cell	[104]
miR-29c	Downregulated	SIRT1	Cultured cells	[105]
miR-34a	Downregulated	CCL22	Human tissues, cultured cells	[106]
miR-99a	Downregulated	PLK1	Human tissues, cultured cells	[107]
	Downregulated	IGF-1R	Human tissues, cultured cells	[108]
miR-100	Downregulated	PLK1	Human tissues, cultured cells	[107]
miR-101	Downregulated	EZH2, EED	Human tissues, cultured cells	[109]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	Mcl1	Cultured cells, human tissues	[110]
	Downregulated	Fos	Human tissues, cultured cells	[111]
miR-122	Downregulated	c-Myc	Human tissues, cultured cells	[112]
	Downregulated		Cultured cells	[113]
	Downregulated	MTTP	Knockout mice	[32]
	Downregulated	IL6, TNF	Knockout mice	[31]
	Downregulated	IGF-1R	Human tissues	[114]
	Downregulated	Cyclin G1	Human tissues, cultured cells	[115]
miR-124	Downregulated	ROCK2, EZH2	Human tissues, cultured cells	[116]
	Downregulated	CDK6, VIM, SMYD3, IQGAP1	Human tissues, cultured cells	[117]
miR-125a/125b	Downregulated		Human tissues, cultured cells	[118]
miR-125b	Downregulated	SUV39H	Human tissues, cultured cells	[119]
	Downregulated	Mcl1, Bclw, IL6R	Human tissues, cultured cells	[120]
	Downregulated		Human tissues, cultured cells	[95]
	Downregulated	PIGF, MMP-2, MMP-9	Human tissues, cultured cells	[121]
	Downregulated	Lin28B	Human tissues, cultured cells	[122]
miR-139	Downregulated	ROCK2	Human tissues, cultured cells	[123]
miR-139-5p	Downregulated		Human tissues, cultured cells	[95]
miR-140-5p	Downregulated	TGFBR1, FGF9	Human tissues, cultured cells	[124]
		DNMT1	Knockout mice	[125]
miR-141	Downregulated	DLC-1	Human tissues	[126]
miR-145	Downregulated		Human tissues	[60]
	Downregulated	IRS1, IRS2, IGF-1R, b-catenin	Human tissues, cultured cells	[127]
	Downregulated		Human tissues	[85]
miR-148a	Downregulated	c-Met	Human tissues, cultured cells	[128]
	Downregulated	HRIP	Mouse xenograft model, cultured cells	[129]
	Downregulated	e-cadherin	Human tissues, cultured cells	[130]
	Downregulated	c-Myc	Cultured cells	[131]
miR-152	Downregulated	DNMT1, GSTP1, CDH1	Human tissues	[132]

Table 2 continued

miRNA	Expression levels	Targets	Main tested samples	References
miR-195	Downregulated	NF-κB pathway	Cultured cells	[133]
	Downregulated	VEGF, VAV2, CDC42	Cultured cells, human tissues	[134]
	Downregulated	Cyclin D1, CDK6, E2F3	Cultured cells, human tissues	[135]
miR-198	Downregulated		Human tissues	[60]
miR-199a/b-3p	Downregulated	PAK4	Human tissues, cultured cells	[30]
miR-199b	Downregulated		Human tissues	[85]
miR-200a	Downregulated	H3 acetylation	Human tissues, cultured cells	[136]
miR-200b	Downregulated		Human tissues, cultured cells	[95]
miR-200c	Downregulated		Human tissues	[82]
miR-200	Downregulated		Human tissues	[82]
miR-203	Downregulated	ABCE1	Human tissues, cultured cells	[117]
miR-214	Downregulated	HDGF	Human tissues, cultured cells	[137]
miR-222	Downregulated		Human tissues	[82]
miR-223	Downregulated	STMN1	Human tissues	[138]
miR-224	Downregulated		Human tissues	[139]
miR-363-3p	Downregulated	c-Myc	Cultured cells	[131]
miR-375	Downregulated	ATG7	Human tissues, cultured cells	[140]
	Downregulated	AEG-1	Human tissues, cultured cells	[141]
miR-429	Downregulated	Rab18	Cultured cells	[142]
miR-449	Downregulated	c-MET	Xenograft, cultured cells	[143]
miR-520e	Downregulated	NIK	Human tissues, cultured cells	[69]
miR-612	Downregulated	AKT2	Cultured cells, human tissues	[144]
miR-637	Downregulated	STAT3 activation	Human tissues, cultured cells	[145]
miR-1271	Downregulated	GLP3	Human tissues, cultured cells	[99]

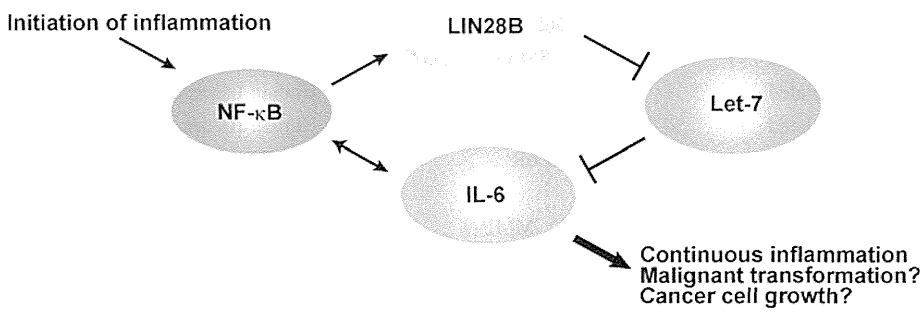

Fig. 2 A model bridging chronic inflammation and transformation by miRNA. Inflammation triggers activation of NF-κB, which leads to transcription of LIN28B. LIN28B inhibits the production of Let-7. Let-7 normally inhibits IL-6 expression, resulting in higher levels of IL-6 than are achieved by NF-κB activation. IL-6 mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop

miRNAs, as a new class of gene expression regulators, may be involved in chronic inflammation-induced carcinogenesis and, in fact, several studies have clarified one such linkage, in which miRNAs may serve as a bridge between continuous inflammation and carcinogenesis.

A flagship report addresses a positive feedback loop of an inflammatory response mediated by NF-κB that activates Lin28B transcription (Fig. 2) [40]. LIN28B, which is an inhibitor of miRNA processing, reduces let-7 levels. Let-7 inhibits IL-6 expression, resulting in higher levels of IL-6 than achieved by NF-κB activation. IL-6-mediated STAT3 activation is necessary for transformation and IL-6 activates NF-κB, completing a positive feedback loop. Although the experiments mainly used MCF10A cells (breast cancer cells), a similar feedback loop was observed in HCC tissues. The authors termed these mechanisms an

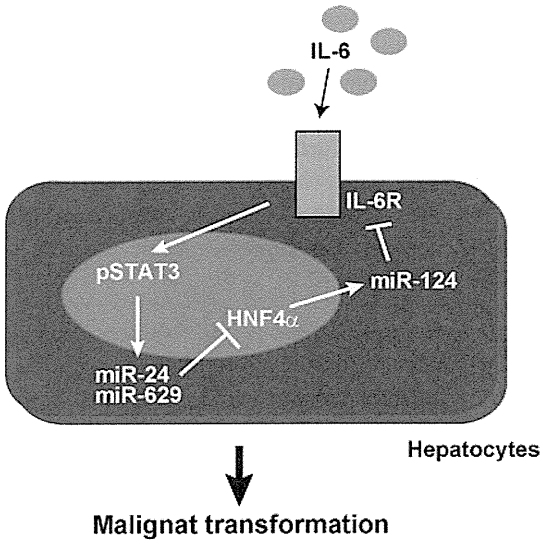

Fig. 3 A model describing a positive feedback loop mediated by miRNAs from transient HNF4α inhibition to transformation. Transient silencing of HNF4α is mediated by miR-24 and miR-629, both of which are induced by STAT3 activation following IL-6 stimulation. miR-124, whose promoter region contains HNF4α-binding sites, targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3, which induces miR-24 and miR-629. This microRNA feedback-inflammatory loop is thought to be crucial in IL-6-mediated liver cancer

"epigenetic switch" because the loop maintains the epigenetic transformed state even in the absence of induction by inflammation (Fig. 2).

Another report addressed hepatocarcinogenesis induced by transient inhibition of HNF4α (Fig. 3) [41]. HNF4α was reported to be involved in liver oncogenesis, although discrepant reports have also been published [42–44]. In that report, transient HNF4α silencing was sufficient to maintain cell transformation. Through a miRNA library screen, miR-24 and miR-629 were identified to target HNF4α. Interestingly, both miRNAs were induced following HNF4α silencing, supporting their involvement in the HNF4α-dependent feedback loop. miR-24 and miR-629 contain the STAT3-binding motif in their promoter region. The authors showed that in response to IL-6, STAT3 binding to their promoters increased, resulting in miRNA expression. They also identified miR-124, whose promoter region contains HNF4α binding sites. miR-124 targets IL-6R and, thus, HNF4α silencing results in reduced expression of miR-124 and enhanced expression of IL-6R and activation of STAT3. The importance of these feedback loops was confirmed in vivo using a mouse HCC model induced by diethylnitrosamine. miR-124 delivery by cationic liposomes prevented tumor development. Thus, these microRNA feedback-inflammatory loops are important and can be a therapeutic target for liver cancer (Fig. 3) [41].

A recent paper reported a similar but distinct observation (Fig. 4). The authors found that when using DEN-induced foci of altered hepatocytes (FAH), LIN28-expressing cells are present in FAH, in which let-7 is down-regulated, resulting in the enhanced expression of IL-6, mediating the progression of malignancies from progenitors. An important difference between the cells in FAH and those in early hepatocarcinogenesis is that IL-6 signaling is autocrine, being mediated by reduced let-7 due to upregulation of LIN28B in FAH cells. This mechanism may contribute to malignant progression from HCC progenitor cells (Fig. 4) [45].

These three reports are from related research groups, and rely on the hypothesis that the IL-6-STAT3 pathway is crucial for hepatocarcinogenesis. Although IL-6 has been implicated as a growth factor in various epithelial cancers [46, 47], its relevance in hepatocarcinogenesis needs to be confirmed to determine the applicability and reproducibility of these findings to the clinical setting.

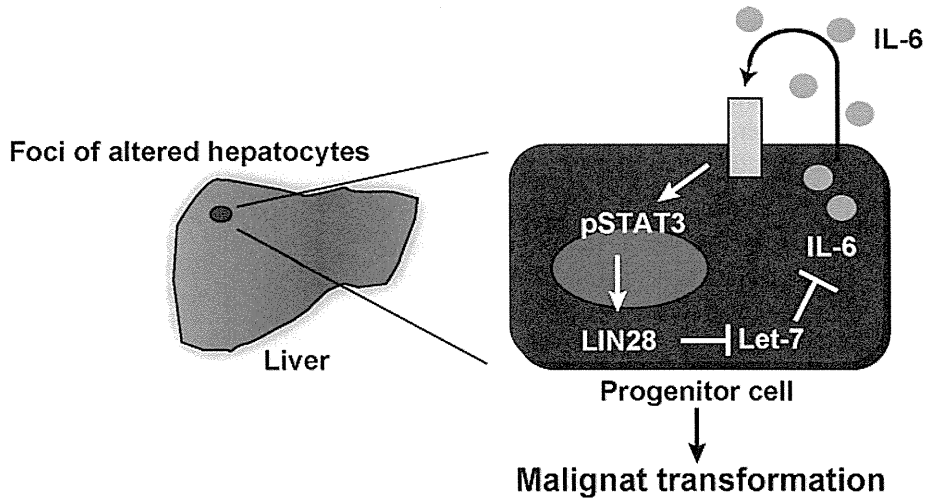

Fig. 4 A model bridging the malignant transformation of precursor cells and autocrine-mediated inflammation by microRNA. LIN28-expressing cells exist in the foci of altered hepatocytes, in which let-7 is downregulated, resulting in enhanced IL-6 expression, which mediates the progression of malignancies from progenitor cells

miRNAs as therapeutic targets in the liver

Recently, miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug for clinical use [48]. It was developed to target HCV, as the stability and propagation of this virus is dependent on a functional interaction between the HCV genome and miR-122 [49, 50]. No harmful events were observed in Phase I studies in healthy volunteers, and Phase II studies proceeded to evaluate the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. The patients were randomly assigned to receive 5 weeks of subcutaneous miravirsen injections at 3, 5 or 7 mg per kg body weight or a placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR-122 binding sites of the HCV genome [48]. The success of miravirsen is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy.

In addition to miravirsen, a clinical trial of MRX34 as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor miR-34 (Mirna Therapeutics, Austin, TX, USA). Further study of MRX34 is being conducted by Mirna Therapeutics, which initiated a Phase I study in May 2013 to examine the effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971). If these oligonucleotide therapies are successful, therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear promising [51].

Issues to be resolved in miRNA involvement in hepatocarcinogenesis

As described above, along with recent discoveries of the diverse effects of miRNAs in hepatocarcinogenesis, miRNA-mediated intervention is promising for the development of new diagnostic, preventive and therapeutic tools. However, the data obtained to date are far from complete. The following are some of the critical issues that we believe need to be resolved.

1. The reason for the non-reproducible results among studies should be determined to utilize the available data more reasonably and efficiently.
2. Identification of crucial driver miRNAs among the diverse deregulated miRNAs is critical to develop useful therapeutics in clinics, although even passive miRNAs may be utilized as markers for diagnosis or prediction of prognosis.
3. Comprehensive target gene analyses using in silico systems biology models should be applied.
4. For effective interventions using miRNA, the delivery method, improved oligonucleotide modification and safety must be further considered. Since miRNAs generally have diverse effects due to targeting multiple mRNAs, undesired outcomes, so called off-target effects, may be encountered, even when a specific miRNA is targeted.

Finding solutions to these issues should be considered as critically important for the near future in order to understand more fully the physiological function of miRNAs in hepatocarcinogenesis and utilize this knowledge in translational research.

Conclusions

The discovery of miRNA has, without doubt, opened up new possibilities for understanding the molecular mechanisms of gene regulation. As numerous findings regarding miRNA, from diverse perspectives, have been reported, the speed of discovery in this field is astonishing. In fact, novel therapeutics targeting miRNAs have already been successfully applied in clinical trials. Some miRNAs may be useful as novel biomarkers. Additionally, the discovery of novel concepts in the pathogenesis of hepatocarcinogenesis frequently involves miRNA. On the other hand, several important issues remain to be resolved in this field. Thus, continuous research in this field is still necessary to develop truly innovative concepts in our understanding of pathogenesis related to miRNA and to transform the obtained knowledge into real clinical applications.

Conflict of interest The authors declare that they have no conflict of interest.

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High ubiquitous mitochondrial creatine kinase expression in hepatocellular carcinoma denotes a poor prognosis with highly malignant potential

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We previously reported the increased serum mitochondrial creatine kinase (MtCK) activity in patients with hepatocellular carcinoma (HCC), mostly due to the increase in ubiquitous MtCK (uMtCK), and high uMtCK mRNA expression in HCC cell lines. We explored the mechanism(s) and the relevance of high uMtCK expression in HCC. In hepatitis C virus core gene transgenic mice, known to lose mitochondrial integrity in liver and subsequently develop HCC, uMtCK mRNA and protein levels were increased in HCC tissues but not in non-tumorous liver tissues. Transient overexpression of ankyrin repeat and suppressor of cytokine signaling box protein 9 (ASB9) reduced uMtCK protein levels in HCC cells, suggesting that increased uMtCK levels in HCC cells may be caused by increased gene expression and decreased protein degradation due to reduced ASB9 expression. The reduction of uMtCK expression by siRNA led to increased cell death, and reduced proliferation, migration and invasion in HCC cell lines. Then, consecutive 105 HCC patients, who underwent radiofrequency ablation with curative intent, were enrolled to analyze their prognosis. The patients with serum MtCK activity >19.4 U/L prior to the treatment had significantly shorter survival time than those with serum MtCK activity ≤19.4 U/L, where higher serum MtCK activity was retained as an independent risk for HCC-related death on multivariate analysis. In conclusion, high uMtCK expression in HCC may be caused by hepatocarcinogenesis *per se* but not by loss of mitochondrial integrity, of which ASB9 could be a negative regulator, and associated with highly malignant potential to suggest a poor prognosis.

Key words: ubiquitous mitochondrial creatine kinase, ankyrin repeat and suppressor of cytokine signaling box protein 9, hepatocellular carcinoma, prognostic factor

Abbreviations: AFP: alpha-fetoprotein; ALT: alanine aminotransferase; ASB: ankyrin repeat and suppressor of cytokine signaling box protein; AST: aspartate aminotransferase; DCP: des-gamma-carboxy prothrombin; GGT: gamma-glutamyltransferase; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; RFA: radiofrequency ablation; ROC: receiver operating characteristic; SOCS: suppressor of cytokine signaling; uMtCK: ubiquitous mitochondrial creatine kinase

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Primary liver cancer, 95% of which is hepatocellular carcinoma (HCC), is ranked third in men and fifth in women as a cause of death from malignant neoplasms in Japan.[1] Furthermore, the worldwide incidence of HCC has increased over several decades, and HCC has recently received considerable attention as a common cause of mortality.[2] HCC often arises in background of liver cirrhosis, which is formed as a result of chronic viral infections, alcoholic injury and some other disorders in the liver.[3,4] Of note, HCC has recently been linked to non-alcoholic fatty liver disease, and this association may contribute to the rising incidence of HCC witnessed in many industrialized countries. It is also problematic that HCC may complicate non-cirrhotic, non-alcoholic fatty liver disease with mild or absent fibrosis, greatly expanding the population potentially at higher risk.[5] Because HCC has a poor prognosis due to its aggressive nature, surgical resection and radiofrequency ablation (RFA) are effective only in early stage of HCC.[4,6] Recurrence occurs almost in 70% of patients with HCC of the first occurrence within 5 years.[7] Regarding the treatment of HCC in United

What's new?

The identification of biomolecules associated with hepatocellular carcinoma (HCC) could greatly improve screening for early disease detection. Ubiquitous mitochondrial creatine kinase (uMtCK) could be a promising marker in this context, though its relevance in HCC is unclear, as it may be associated with mitochondrial stability rather than carcinogenesis. Here, in transgenic mice susceptible to the loss of liver mitochondrial integrity, uMtCK was found to be elevated in HCC tissue but not in non-tumorous liver tissue. Increased uMtCK was further linked to reduced expression of ASB9 and elevated risk for HCC-related death.

States veterans, approximately 40% of patients were reportedly diagnosed during hospitalization. Most patients were not seen by a surgeon or oncologist for treatment evaluation and only 34% received treatment.[8] Although there was no effective chemotherapy for advanced HCC for a long time, a novel anti-cancer therapy such as anti-angiogenesis pathway therapy has just recently been developed to prolong survival in patients with the advanced disease.[9,10] However, its effect is rather limited, just extending median survival from 7.9 months to 10.7 months in patients with advanced HCC.[10] Thus, the effective way for early detection of HCC is urgently needed. To this end, the recommended screening strategy for patients with cirrhosis includes the determination of serum alpha-fetoprotein (AFP) levels and an abdominal ultrasound every 6 months to detect HCC at an earlier stage. AFP, however, is a marker characterized by poor sensitivity and specificity.[11] Although other potential markers such as des-gamma-carboxy prothrombin (DCP) and squamous cell carcinoma antigen-immunoglobulin M complex have been proposed to use for diagnosis of HCC, none of them is optimal; however, when used together, their sensitivity in detecting HCC is increased.[11–14] For cholangiocarcinoma, which is a relatively rare type of primary liver cancer that originates in the bile duct epithelium, carbohydrate antigen 19-9, carcinogenic embryonic antigen and cancer antigen 125 have shown sufficient sensitivity and specificity to detect and monitor it. In particular, the combination of these markers seems to increase their efficiency in diagnosing of cholangiocarcinoma.[15]

In this context, we have recently reported that serum mitochondrial creatine kinase (MtCK) activity is increased in patients with HCC, even in those with early stage, suggesting that MtCK may be useful to detect early stage of HCC.[16] Among two tissue-specific isozymes of MtCK, that is, ubiquitous MtCK (uMtCK) and sarcomeric MtCK, we have found that the increase in serum MtCK activity in HCC patients was mostly due to that in serum uMtCK activity but not in serum sarcomeric MtCK activity.[16] Then, we have further observed the higher expression of uMtCK mRNA in HCC cell lines than in normal human liver tissues.[16] Of note, the increased uMtCK expression occurred not only upon malignant changes in the liver, but also in several other malignant tumors such as gastric cancer, breast cancer and lung cancer, where the high expression of uMtCK suggests a poor prognosis.[17–19] In contrast, uMtCK was down-regulated in oral squamous cell carcinoma,[20] and sarcomeric MtCK was also down-regulated during sarcoma development in leg muscle in mice.[21] Therefore, we aimed to elucidate the mechanism(s) and the significance of high uMtCK expression in HCC in this study.

We first examined whether loss of mitochondrial integrity might be involved in high uMtCK expression in HCC, using hepatitis C virus (HCV) core gene transgenic mice. HCV core protein has been first demonstrated to play a pivotal role in HCC development within these transgenic mice, which are known to lose mitochondrial integrity and subsequently develop HCC without apparent inflammation and fibrosis in the liver.[22,23] As a regulatory factor for uMtCK expression, we have focused on the ankyrin repeat and suppressor of cytokine signaling (SOCS) box protein (ASB) family, which reportedly plays an important role in biological processes and regulations of cell proliferation and differentiation. The ASBs have two functional domains: a SOCS box and a variable number of N-terminal ankyrin repeats. Although SOCS domain uses the SH2 domain to recruit substrates, the ankyrin repeat regions serve as a specific protein–protein interaction domain to recruit target substrates.[24] One of ASB family protein, ASB9, was found to interact with brain type of creatine kinase, leading to its degradation.[25] Recently, uMtCK was found to be another ASB9 target.[26] Ankyrin repeat domains of ASB9 associates with the substrate binding site of uMtCK and induce its ubiquitination. Thus, we analyzed the potential association between uMtCK and ASB9 in HCC cell lines, HepG2, PLC/PRF/5, HuH7, in which the expression of uMtCK mRNA was shown to be increased compared with normal liver tissues.[16] To clarify the significance of high uMtCK expression in HCC, we used the siRNA approach to silence uMtCK expression and study its effects on HCC cell lines. Finally, we analyzed the clinical significance of high uMtCK expression in HCC patients who were treated with RFA.

Material and Methods

Materials

Human normal liver RNA was purchased from Cell Applications (San Diego, CA), and human whole liver cell pellets from DV Biologics (Costa Mesa, CA). Specific antibodies against uMtCK and ASB9 were obtained from Abcam (Cambridge, UK), an antibody against caspase 3 from Cell Signaling Technology (3G2; Boston, MA), and an antibody against beta-actin from Sigma–Aldrich (MO).

Cells and cell culture

HCC cell lines, HepG2 and PLC/PRF/5 were obtained from RIKEN BioResource Center (Tsukuba, Ibaraki, Japan) and HuH7 from Health Science Research Resources Bank, Japan Health Science Foundation. HepG2 and PLC/PRF/5 were maintained in RPMI-1640 containing 10% of fetal bovine serum, and HuH7, in Dulbecco's Modified Eagle Medium containing 10% of fetal bovine serum.

Transgenic mice

HCV core gene transgenic mice were produced as previously described.[22] Nontransgenic littermates of the transgenic mice were used as controls. All mice were fed a standard pelleted diet and water *ad libitum* under normal laboratory conditions of 12 hr-light/dark cycles, and received humane care. The experimental protocol was approved by Animal Research Committee of the University of Tokyo.

Quantitative real-time PCR

Total RNA of HCC cell lines (HepG2, PLC/PRF/5 and HuH7), human normal liver and livers from non-transgenic and HCV core gene transgenic mice were extracted using TRIZOL reagent (Invitrogen, CA). One microgram of purified total RNA was transcribed using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with a LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Diagnostics, CA) or TaqMan Universal Master Mix. The primer pairs used were as follows: human ASB9: 5′-CCTGGCATCAGGCTTCTTTC-3′ and 5′-ACCCCTGGCTGATGAGGTTC-3′[27]; human beta-actin: 5′-GGGTCAGAAGGATTCCTATG-3′ and 5′-CCTTAATGTC ACGCACGATTT-3′.[26] Mouse uMtCK primers and probe were obtained from Applied Biosystems, TaqMan Gene Expression Assays (Mm00438221_m1). The samples were incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. The target gene mRNA expression level was relatively quantified to beta-actin using $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, User Bulletin No 2).

ASB9 transfection

Cells, transiently expressing human ASB9 protein, were constructed using mammalian cell expression vector p3FLAG CMV-10 containing the corresponding cDNA which derived from human normal liver RNA. The primers used for cloning were 5′-GCGGATCCGTCATGGATGGCAAACAAGGG-3′ and 5′-GAGCGGCCGCTTAAGATGTAGGAGAAACTGTT T-3′ which were designed based on human ASB9 reference sequence (NM_001031739.2). The ASB9 cDNA was created by PCR and verified by DNA sequencing.

Immunoblot analysis

Cell and tissue extracts were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, IL) plus Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Immunoblot analysis was performed as previously described,[28] using NuPAGE SDS-PAGE Gel (Invitrogen) and iBlot Dry Blotting System (Invitrogen) with specific antibodies against uMtCK (dilution 1:1,000), ASB9 (dilution 1:500), caspase 3 (dilution 1:1,000) and beta-actin (dilution 1:2,000). Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare, Buckinghamshire, UK), and recorded using a LAS-4000 image analyzer (Fuji Film, Tokyo, Japan). The intensities of immunodetected bands were quantified with NIH Image J software.

uMtCK siRNA transfection

Cells were transfected with the human uMtCK-specific 23/27mer RNA duplex or a universal negative control duplex at 20 nM, respectively, according to the vender instructions (Integrated DNA Technologies, IA). The human uMtCK-specific RNA duplex used was 5′-UGAAGCACACCACGGAUCU-3′ and 3′-ACUUCGUGUGGUGCCUAGA-5′,[29] negative control RNA duplex, 5′-CGUUAAUCGCGUAUAAUACGCGUAT-3′ and 3′-CAGCAAUUAGCGCAUAUUAUGCGCAUA-5′ (Integrated DNA Technologies). The transfection was performed using Lipofectamine Plus™ (Invitrogen) as described.[29]

Cell membrane integrity and proliferation assays

Cell membrane integrity was determined using the In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma–Aldrich). HCC cell lines were inoculated in six-well plates at 2.5×10^5 cells/well and cultured for 24 hr before uMtCK siRNA or universal negative control transfection. Dead cells were assessed at 48 hr after transfection.

Cell proliferation in HCC cell lines was measured at 48 hr after transfection with uMtCK siRNA or universal negative control by determination of BrdU incorporation using the Cell Proliferation ELISA, BrdU colorimetric assay (Roche Applied Science, Upper Bavaria, Germany). In the above two assays, absorbance was measured by plate reader (SPECTRA Thermo, TECAN, Männedorf, Switzerland).

Cell migration and invasion assays

Cell migration and invasion assays were performed according to the vender's instruction (BD, NJ). Cells transfected with uMtCK siRNA or universal negative control were cultured for 24 hr, then 2×10^4 cells were plated into the upper chamber of 24-well plates with 8 μm of pore size in serum-starved condition to examine cell migration and polycarbonate transwell filter chamber coated with Matrigel (BD BioCoat Matrigel Invasion Chamber) to check cell invasion. In both assays, 750 μL medium supplemented with 10% serum was added into the lower chambers. Cells were incubated at 37°C for 22 hr, and the inside chambers were removed with cotton swabs and cells that had transferred to the lower membrane surface were fixed and stained with Diff-Quik stain. Cell counts (four random 100× fields per well) are expressed as the mean number of cells per field of view.

Patients and measurement of MtCK activity

Consecutive 147 HCC patients with cirrhosis caused by hepatitis B virus or HCV, who were admitted into the Department of Gastroenterology, the University of Tokyo Hospital, Tokyo, Japan, between January and April 2010, were previously enrolled to analyze serum MtCK activity.[16] Diagnosis of cirrhosis was based on the presence of clinical and laboratory features indicating portal hypertension, and diagnosis of HCC was made by dynamic CT or MRI.[30,31] Prior to the treatment of HCC, serum MtCK activity was measured[16] with an immuno-inhibition method using the two types of anti-MtCK monoclonal antibodies.[32] Among these patients, 105 patients, who had been successfully treated by RFA without residual HCC after the treatment, were enrolled in the current prognosis analysis. The detailed procedure of RFA has been meticulously described elsewhere.[33] Overall survival of these 105 patients was analyzed from the time of measurement of serum MtCK activity to death related to HCC, excluding the death not associated with HCC expansion or liver insufficiency, such as cardiovascular events or other organ malignancy, or to March 2013.

This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions. A written informed consent was obtained for the use of the samples in this study.

Statistical analysis

The results of *in vitro* experiments are expressed as the means and standard error of the mean. Student's *t* test (two tailed) was used for comparison unless indicated otherwise. The results were considered significant when *p*-values were 0.05. In the analysis of risk factors for HCC-related death, we tested the following variables obtained at the time of entry on the univariate and multivariate Cox proportional hazard regression analysis: age, sex, hepatitis B infection, serum MtCK activity, serum albumin concentration, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, total bilirubin concentration, AFP concentration, DCP concentration, platelet count, prothrombin activity and liver stiffness values. Survival and recurrence curves were created using Kaplan–Meier method and compared *via* log-rank test. Data processing and analysis were performed using S-PLUS 2000 (MathSoft, Seattle, WA) and SAS Software version 9.1 (SAS Institute, Cary, NC).

Results

Loss of mitochondrial integrity may not contribute to high expression of uMtCK in HCC

Mutations of mitochondrial DNA have been reported to be involved in hepatocarcinogenesis in humans.[34,35] Furthermore, in a mouse model for hepatocarcinogenesis, oxidative stress was shown to lead to loss of mitochondrial integrity in the liver and ultimately hepatocarcinogenesis.[23] Thus, we wondered whether loss of mitochondrial integrity in the liver might be associated with increased expression of uMtCK in HCC. To examine this idea, we used a transgenic mouse model of HCC in HCV infection (transgenic line S-N/863), with which the direct association between HCV and HCC was first described.[22] In these HCV core gene transgenic mice, loss of mitochondrial integrity has been reported to be observed as early as 2 months of age and increased in an age-dependent manner,[23] and ultimately HCC develops at 19 months of age without apparent inflammation or fibrosis in the liver.[22]

We examined uMtCK mRNA levels in the liver of these HCV core protein transgenic mice at 6 months and 19 months of age. These mice at 6 months of age reportedly develop hepatic steatosis[22] as well as loss of mitochondrial integrity.[23] In these mice at 19 months of age, tumor tissues of HCC and non-tumorous tissues of the liver were analyzed. Non-transgenic mice at 6 months of age were used as control. uMtCK mRNA levels were increased in tumor tissues of HCC in HCV core gene transgenic mice at 19 months of age by 7.7-fold compared to the liver tissues of control mice ($p = 0.02$; Fig. 1*a*). In these HCV core transgenic mice at 19 months of age, uMtCK protein expression was detected in HCC tissues but not in non-tumorous tissues by immunoblot analysis (Fig. 1*b*). These results suggest that hepatocarcinogenesis *per se* but not loss of mitochondrial integrity may contribute to the increase in uMtCK levels in HCC.

Transient expression of ASB9 negatively regulates uMtCK protein levels in HCC cells

It has been reported that ASB protein family is importantly involved in ubiquitination-mediated proteolysis pathway and each member of this large protein family has a different target to be degraded. In ASB protein family, we paid attention to ASB9, which reportedly plays a crucial role in the regulation of the brain type of creatine kinase and uMtCK. HCC cell lines, HepG2, PLC/PRF/5 and HuH7, were selected for *in vitro* experiments, because they had been reported to express high levels of uMtCK mRNA compared to human normal liver tissue.[16] To study whether ASB9 could regulate uMtCK protein levels in these HCC cells, we first measured ASB9 mRNA expression in those cells. Figure 2*a* demonstrates the low ASB9 mRNA expression in HCC cell lines, contrasting with high uMtCK mRNA expression levels in those cells.[16] In line with our mRNA expression data, ASB9 protein levels were almost undetectable in HepG2, PLC/PRF/5 and HuH7 cells comparing to normal whole liver cell pellets (Fig. 2*b*). Further, we investigated the effect of transient overexpression of ASB9 on uMtCK protein levels in HepG2, PLC/PRF/5 and HuH7 cells. Cells were transiently transfected with mammalian cell expression vector p3FLAG-CMV10 containing human ASB9 DNA and harvested at 36 hr after transfection to analyze protein levels. Down-regulation of uMtCK protein levels by transient

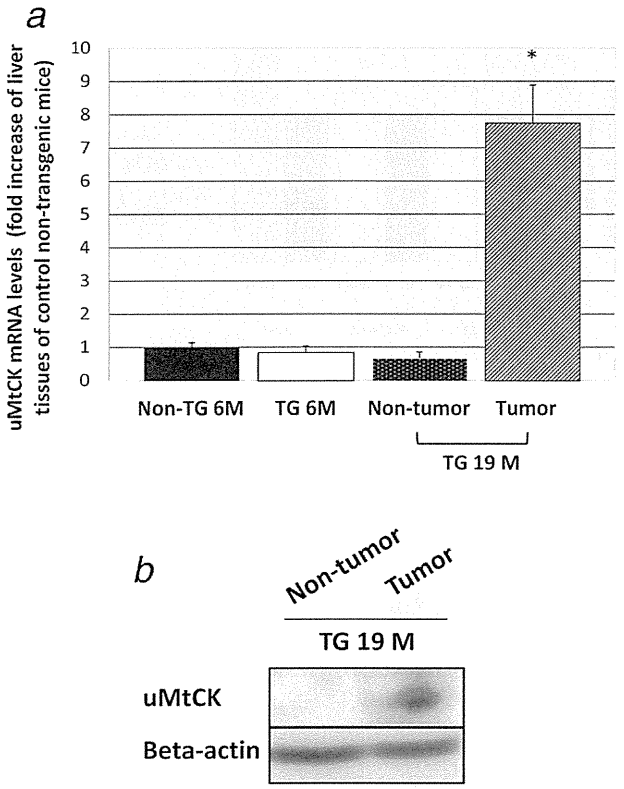

Figure 1. uMtCK mRNA and protein levels in liver tissues of the control non-transgenic, HCV core gene transgenic mice. (*a*) uMtCK mRNA levels were examined by real-time PCR in liver tissues of the control non-transgenic mice (Non-TG) at 6 months of age ($n = 4$), and HCV core gene transgenic mice (TG) at 6 ($n = 4$) and 19 months of age ($n = 4$). For HCV core gene transgenic mice at 19 months of age, HCC tissues and non-tumorous tissues were separately evaluated. Results represent a fold increase level of liver tissues of control non-transgenic mice. An asterisk indicates a significant difference ($p = 0.02$) from liver tissues of non-transgenic mice. (*b*) uMtCK protein levels were analyzed by immunoblotting in HCC tissues and non-tumorous tissues in the livers of HCV core gene transgenic mice at 19 months of age.

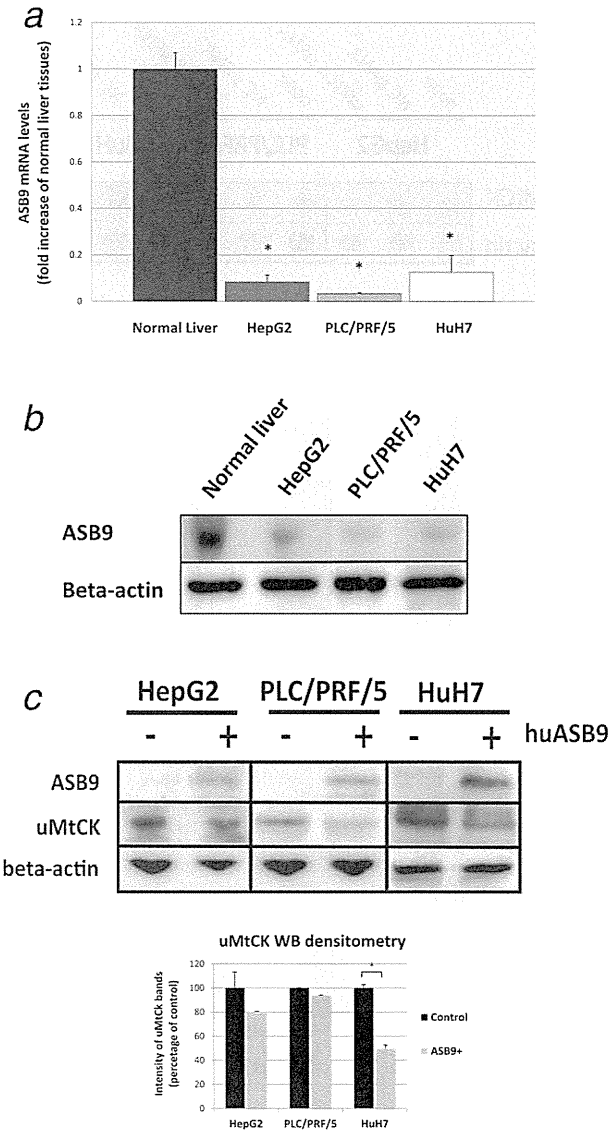

Figure 2. ASB9 expression and the effect of ASB9 transfection on uMtCK protein levels in HCC cells. ASB9 mRNA (*a*) and protein (*b*) levels in HepG2, PLC/PRF/5 and HuH7 cells were examined by real-time PCR and immunoblot analysis, respectively. As a positive control for ASB9 mRNA and protein expressions, human normal liver RNA and human whole liver cell pellets were used. An asterisk indicates a significant difference from normal liver tissue; $p = 0.006$ for HepG2, $p = 0.005$ for PLC/PRF/5 and $p = 0.01$ for HuH7. Increased expression of ASB9 by transfection caused reduced protein levels of uMtCK in HepG2, PLC/PRF/5 and HuH7 cells (*c*). An asterisk indicates a significant difference ($p = 0.007$) from control without ASB9 transfection.

overexpression of ASB9 was observed significantly in HuH7 cells ($p = 0.007$), and a trend of decreased uMtCK protein levels was found in HepG2 and PLC/PRF/5 cells, although not statistically significant (Fig. 2*c*). These results suggest a functional interaction of ASB9 with uMtCK may lead to degradation of uMtCK protein in HCC cell lines, as previously described.[26]

Reduction in uMtCK expression led to increased cell death, and reduced proliferation, migration and invasion of HCC cells

To inhibit high uMtCK expression in HepG2, PLC/PRF/5 and HuH7 cells,[16] isoform-specific siRNA was chosen as described[29] and successfully silenced target protein expression; the results from immunoblot analysis of untransfected and transfected cell lysates with universal negative control and uMtCK siRNA are shown in Figure 3*a*. As expected, in all HCC cell lines transfected with uMtCK siRNA, the expression levels of uMtCK were clearly reduced at 36 hr after transfection (Fig. 3*a*).

Then, the effects of a reduction in uMtCK expression on cell membrane integrity and proliferation were determined in HepG2, PLC/PRF/5 and HuH7 cells. In the first step, we have checked cell membrane integrity by measuring lactate

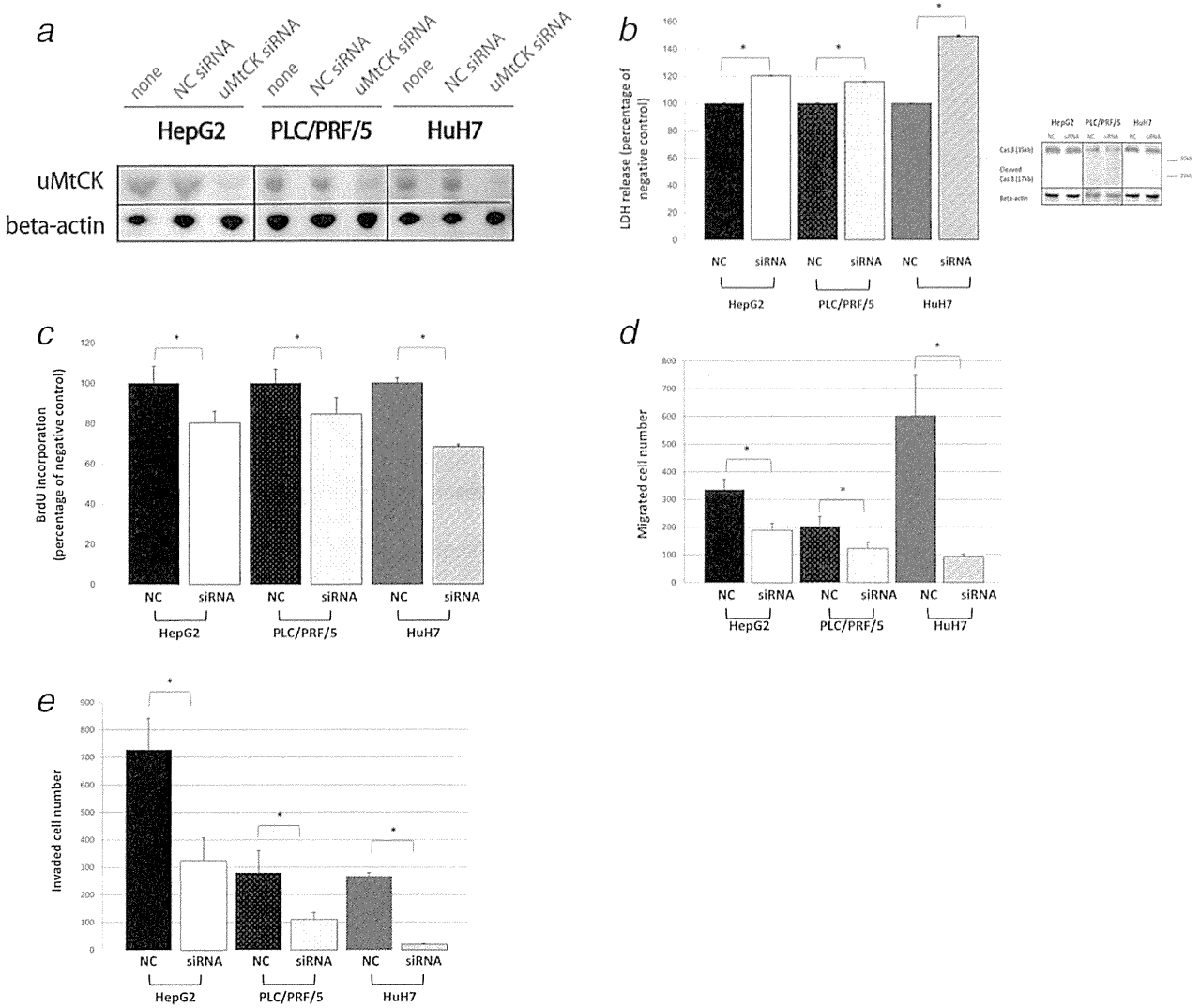

Figure 3. Increase in cell death and reduction in proliferation, migration and invasion by reduced uMtCK expression with siRNA in HCC cell lines. Human HCC cell lines, HepG2, PLC/PRF/5 and HuH7 cells, were transfected with 20 nM uMtCK siRNA or universal negative control, and uMtCK levels were examined by immunoblot analysis. None, no transfection; NC, negative control (*a*). Cell death (*b*), proliferation (*c*), migration (*d*) and invasion (*e*) were assessed in these HCC cell lines treated with or without uMtCK siRNA. An asterisk indicates a significant difference; $p < 0.001$ for cell death and proliferation, $p < 0.02$ for cell migration and invasion from NC.

dehydrogenase released into the culture medium in universal negative control- and uMtCK siRNA-transfected cells (Fig. 3*b*). In all three cells, transfection with uMtCK siRNA led to an increase in the rate of cell lysis by 20.3% in HepG2, by 15.9% in PLC/PRF/5 and by 49.2% in HuH7, compared to respective control cells transfected with universal negative control ($p < 0.001$). However, caspase 3 activity was not altered in uMtCK siRNA-transfected cells compared to universal negative control-transfected cells (Fig. 3*b*), suggesting that lactate dehydrogenase release may be explained by some non-specific cell lysis but not by programmed cell death.

Next, to examine a potential association of the reduction in uMtCK expression with cell proliferation rate, BrdU incorporation assay was performed (Fig. 3*c*). A reduction in cell proliferation was detected in all three HCC cell lines by 19.8% in HepG2, by 15.5% in PLC/PRF/5 and by 31.7% in HuH7, compared to respective control cells transfected with universal negative control ($p < 0.001$). These results suggest that high expression of uMtCK may play a role in sustaining active proliferation of HCC cells.

The ability of a cancer cell to undergo migration and invasion allows the cell to change position within the tissues. To spread within the tissues, tumor cells use migration and invasion mechanisms. Thus, we investigated the effects of uMtCK inhibition on HCC cell migration and invasion by conducting assays for Matrigel-coated chamber migration and invasion. As shown in Figure 3*d*, silencing of uMtCK decreased migration rate by 44.1% in HepG2, by 40.0% in

Table 1. Baseline characteristics

Parameter	*N* = 105
Age (year)[1]	70.7 ± 6.7 (49–84)
Male[2]	63 (60.0)
Hepatitis B/C	8 / 97
MtCK (U/L)[3]	9.71 (5.99–19.44)
Albumin (g/dL)[3]	3.4 (3.1–3.9)
AST (U/L)[3]	55 (35–76)
ALT (U/L)[3]	45 (26–60)
GGT (U/L)[3]	37 (28–62)
Total bilirubin (mg/dL)[3]	0.9 (0.7–1.3)
AFP (ng/dL)[3]	18 (8–66)
DCP (mAU/mL)[3]	26 (17–58)
Platelet ($\times 10^4/\mu L$)[3]	9.3 (6.3–11.7)
Prothrombin time (sec)[3]	12.1 (11.5–13.1)
Liver stiffness (kPa)[3]	26.3 (18.8–42.2)

[1]Data were expressed as mean ± SD (range).
[2]Data were expressed as number (%).
[3]Data were expressed as median (first to third quartile).

PLC/PRF/5 and by 84.1% in HuH7 cells in comparison with the universal negative control-transfected cells ($p < 0.02$). Furthermore, the results from Matrigel invasion assay indicate that the reduction of uMtCK expression by siRNA transfection inhibited the invasion of HepG2, PLC/PRF/5 and HuH7 cells by 51.7, 62.6 and 92.4%, compared to the universal negative control-transfected cells ($p < 0.02$) (Fig. 3*e*). Collectively, high expression of uMtCK may contribute to active migration and invasion of HCC cells.

HCC patients with higher serum MtCK activity had a poorer prognosis after RFA

Because above *in vitro* results using HCC cell lines suggest that HCC cells with higher expression of uMtCK may have more malignant potential, we next examined a potential association between serum MtCK activity and prognosis in patients with HCC. As described earlier, among two tissue-specific isozymes of MtCK, that is, uMtCK and sarcomeric MtCK, the increase in serum MtCK activity in HCC patients was mostly due to that in serum uMtCK activity but not in serum sarcomeric MtCK activity.[16] To this end, a prognosis of HCC patients, who had been previously enrolled to examine their serum MtCK activity and successfully treated by RFA without residual HCC after the treatment, was analyzed. Characteristics of these 105 HCC patients are shown in Table 1. During the mean follow-up period of 848 days, HCC-related death was observed in 17 patients. First, to evaluate the potential ability of MtCK values to predict survivals or death, a receiver operating characteristic (ROC) curve was generated. The ROC curve showed that a MtCK cutoff of 19.4 U/L had a sensitivity of 76.9% and a specificity of 83.8% for discriminating survivors and deceased patients (Fig. 4*a*). Then, Figure 4*b* shows the actuarial survival curves of these patients subdivided according to their serum MtCK activity prior to RFA for HCC, that is, ≤19.4 U/L and >19.4 U/L; overall survival was shorter in patients with serum MtCK activity >19.4 U/L than in those with ≤19.4 U/L ($p = 0.0002$; log-rank test; Fig. 4*b*). Then, risk factors for HCC-related death were analyzed. On the univariate analysis, high serum MtCK activity (>19.4 U/L) was a significant risk factor for HCC-related death (Table 2). Other significant risk factors for HCC-related death included serum albumin concentration, serum AST levels, serum total bilirubin concentration, platelet count and prothrombin time (Table 2). Then, multivariate Cox proportional hazard regression analysis revealed that serum MtCK activity >19.4 U/L was an independent risk for HCC-related death, with a hazard ratio of 2.32 (95% confidence interval: 1.03–5.25; $p = 0.042$; Table 2). Serum albumin concentration and serum AST levels were also independently associated with HCC-related death (Table 2). Regarding recurrence, HCC in patients with serum MtCK activity >19.4 U/L recurred earlier than HCC in those with serum MtCK activity ≤19.4 U/L, as depicted in Figure 4*c* ($p = 0.004$; log-rank test); median (interquartile range) time to recurrence was 189 (107–292) days in patients with serum MtCK activity >19.4 U/L, whereas 278 (160–445) days in those with serum MtCK activity ≤19.4 U/L. Collectively, these findings suggest that HCC patients with higher serum MtCK activity may have shorter survival time possibly due to more malignant potential.

Discussion

Little is known about whether there might be an association between the status of mitochondria and uMtCK expression. Kwon *et al.* have reported that ASB9 negatively regulated uMtCK expression with the inhibition of mitochondrial function,[26] suggesting that low uMtCK expression could be associated with loss of mitochondrial integrity. There could be several possibilities regarding the status of mitochondria and uMtCK expression in the liver or HCC; one is that loss of mitochondrial integrity might be associated with reduced uMtCK expression as previously reported.[26] As another possibility, uMtCK expression might be increased as a compensatory mechanism with loss of mitochondrial integrity. In fact, this is exactly the case with sarcomeric MtCK in mitochondrial myopathies.[36] It is also possible that there might be no association in general between loss of mitochondrial integrity and uMtCK expression. In this context, we wondered whether loss of mitochondrial integrity in the liver might be involved in the mechanism of increased uMtCK expression in HCC. To examine this, HCV core gene transgenic mice were used, because these mice develop HCC with loss of mitochondrial integrity in the liver in the absence of inflammation and fibrosis.[22,23] As a result, uMtCK expression was essentially not altered in non-tumorous liver tissues with loss of mitochondrial integrity but clearly enhanced in HCC tissues, suggesting that hepatocarcinogenesis *per se* but not

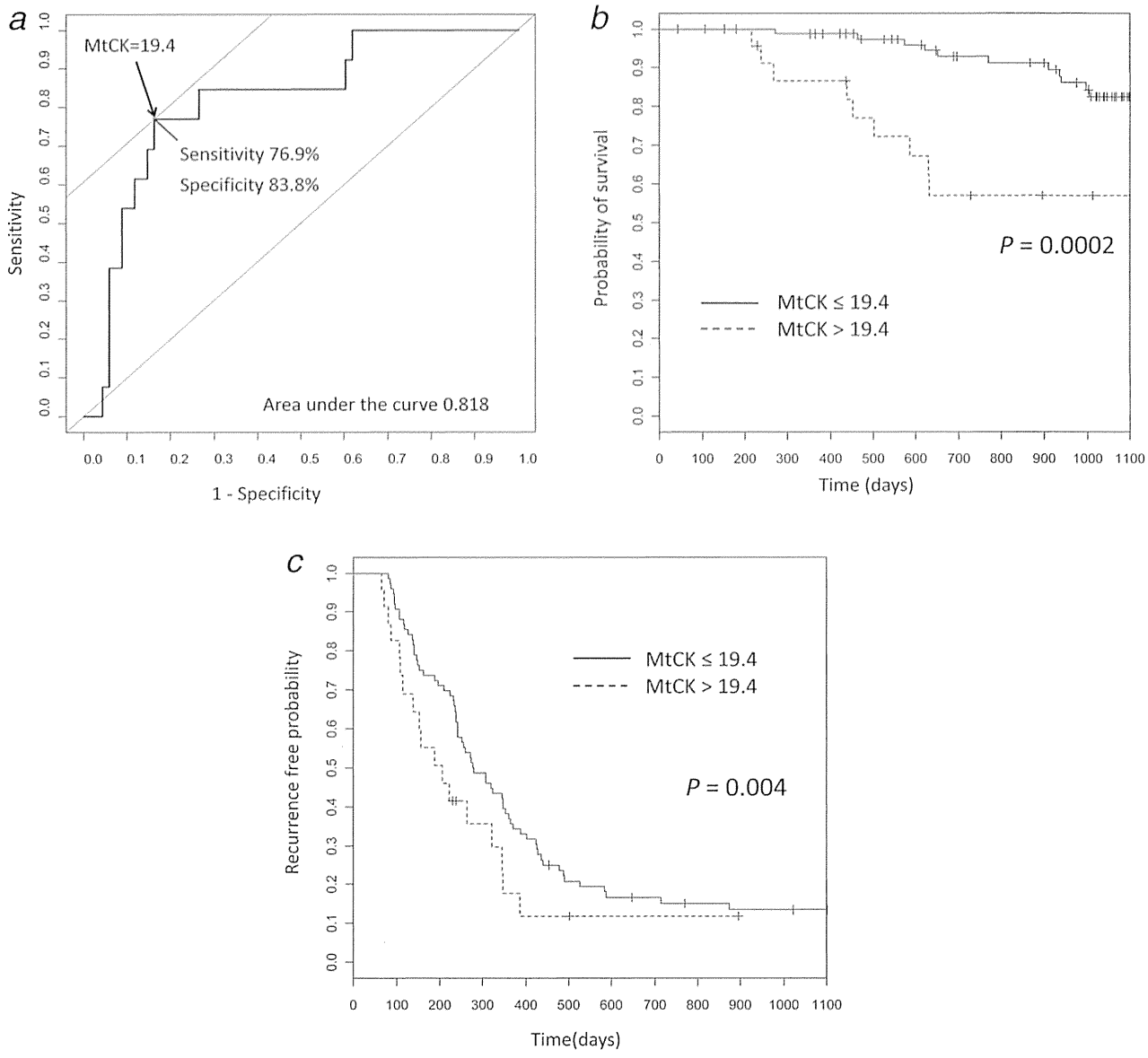

Figure 4. (*a*) ROC curve showing the overall accuracy of serum MtCK activity for discriminating between survivors and deceased patients. The arrow identifies the best cutoff value (*i.e.*, 19.4 U/L) of serum MtCK activity. Kaplan–Meier survival (*b*) and recurrence (*c*) curve of the studied patients subdivided according to their serum MtCK activity prior to RFA for HCC. Solid line, ≤19.4 U/L; dashed line, >19.4 U/L.

loss of mitochondrial integrity may contribute to increased uMtCK expression in HCC.

Regarding the regulatory mechanism(s) of increased uMtCK expression in HCC, we have found that ASB9 interacted with uMtCK to reduce its protein levels in HCC cells, similarly to HEK293 cells as previously described.[26] In normal liver, uMtCK levels are generally at a very low level, while sarcomeric MtCK as a muscle-specific isoform is not expressed at all,[37] whereas ASB9 mRNA expression is reportedly abundant.[26] Thus, ASB9 may play a physiological role to keep uMtCK protein levels low in the liver. Regarding HCC, ASB9 mRNA expression in HCC cells were much lower than that in normal liver tissue in the current study. This finding raises the possibility that low expression of ASB9 may explain, at least in part, high protein levels of uMtCK in HCC. Collectively, we may suggest that the two possible mechanisms of increased uMtCK protein levels in HCC cells should be increased gene expression and decreased protein degradation due to reduced ASB9 expression. It has been reported that colorectal cancer with low ASB9 expression may have a higher malignant potential and a poorer prognosis than that with high ASB9 expression,[27] suggesting a negative association of ASB9 with uMtCK protein levels also in colorectal cancer cells. Nonetheless, a potential role of ASB9 in the regulation of uMtCK expression in HCC *in vivo* should be further elucidated.

Table 2. Risk factors for HCC-related death evaluated by univariate/multivariate Cox proportional hazard regression

	Univariate		Multivariate	
Parameter	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Age (year)	1.02 (0.95–1.10)	0.60		
Female	1.45 (0.56–3.77)	0.44		
Hepatitis B	1.37 (0.18–10.3)	0.76		
MtCK >19.4 (U/L)	5.03 (1.93–13.1)	<0.001	2.32 (1.03–5.25)	0.042
Albumin	0.15 (0.05–0.44)	<0.001	0.26 (0.09–0.71)	0.009
AST	1.02 (1.01–1.03)	<0.001	1.01 (1.00–1.02)	0.028
ALT	1.01 (0.99–1.02)	0.13		
GGT	1.00 (0.98–1.01)	0.45		
Total bilirubin	3.23 (1.98–5.29)	<0.001	1.72 (0.97–3.04)	0.064
AFP >100 (ng/dL)	2.28 (0.84–6.18)	0.11		
DCP >80 (mAU/mL)	2.74 (0.99–7.45)	0.59		
Platelet	0.83 (0.71–0.97)	0.017	0.89 (0.76–1.04)	0.14
Prothrombin time	1.32 (1.11–1.57)	0.002	0.91 (0.70–1.17)	0.45
Liver stiffness	1.02 (0.98–1.04)	0.25		

Reduction of uMtCK expression in HCC cells led to the inhibition in their proliferation, migration and invasion. The similar effects of inhibition of uMtCK expression were reported in Hela cells[29] and breast cancer cells.[17] This finding may be in agreement with the notion that the creatine kinase system is generally essential for the control of cellular energetics in tissues or cells with high and fluctuating energy requirements.[37] Indeed, overexpression has been reported for different creatine kinase isoforms in different types of cancer and has provided a more general growth advantage to solid tumors.[37,38] Overexpression of uMtCK in different Hodgkin-derived cell lines has been described as a marker for poor prognosis.[39] Increased uMtCK levels in cancer cells might be a part of metabolic adaptation of those cells to perform high growth rate under oxygen and glucose restriction as typical for many cancers; it could help to sustain energy turnover, but would be also protective against stress situations such as hypoxia and possibly protect cells from death.[40] Nonetheless, these *in vitro* findings raise the possibility that high expression of uMtCK in HCC may be associated with its active growth and metastasis.

Then, we performed a follow-up study of the HCC patients, with whom we showed the increased serum MtCK activity.[16] Among the entire HCC patients in the previous study, we enrolled the patients who underwent RFA with curative intent to examine the potential association between serum MtCK activity and prognosis in this study. In the previous report, serum MtCK activity was also enhanced in the patients with liver cirrhosis compared to healthy control, although less prominent than in those with HCC and liver cirrhosis,[16] suggesting that background liver status of HCC may also affect serum MtCK activity. In this context, because RFA with curative intent was performed on patients without advanced liver damages such as high serum total bilirubin concentration, low platelet counts or massive ascites,[33] the potential association between serum MtCK activity and prognosis of HCC patients could be assessed with less bias from background liver status. Furthermore, of note, HCC patients treated with RFA had no extended tumor lesions, that is, three or fewer lesions, each 3.0 cm in diameter.[33] As a result, the HCC patients with higher serum MtCK activity had a significantly poorer prognosis than those with lower serum MtCK activity on a survival analysis, and higher serum MtCK activity was retained as a significant risk for HCC-related death on multivariate analysis. Thus, in line with the current *in vitro* findings, it is suggested that HCC with increased uMtCK expression may have highly malignant potential.

In conclusion, high uMtCK expression in HCC may be caused by hepatocarcinogenesis *per se* but not by loss of mitochondrial integrity, and associated with highly malignant potential, where ASB9 could be one of the regulators of uMtCK expression. In the clinical setting, higher serum MtCK activity was associated with a poorer prognosis of HCC, suggesting that HCC with high serum MtCK activity should be thoroughly treated when considered to be curative.

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Loss of liver E-cadherin induces sclerosing cholangitis and promotes carcinogenesis

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E-cadherin is an important adhesion molecule whose loss is associated with progression and poor prognosis of liver cancer. However, it is unclear whether the loss of E-cadherin is a real culprit or a bystander in liver cancer progression. In addition, the precise role of E-cadherin in maintaining liver homeostasis is also still unknown, especially in vivo. Here we demonstrate that liver-specific E-cadherin knockout mice develop spontaneous periportal inflammation via an impaired intrahepatic biliary network, as well as periductal fibrosis, which resembles primary sclerosing cholangitis. Inducible gene knockout studies identified E-cadherin loss in biliary epithelial cells as a causal factor of cholangitis induction. Furthermore, a few of the E-cadherin knockout mice developed spontaneous liver cancer. When knockout of E-cadherin is combined with Ras activation or chemical carcinogen administration, E-cadherin knockout mice display markedly accelerated carcinogenesis and an invasive phenotype associated with epithelial–mesenchymal transition, up-regulation of stem cell markers, and elevated ERK activation. Also in human hepatocellular carcinoma, E-cadherin loss correlates with increased expression of mesenchymal and stem cell markers, and silencing of E-cadherin in hepatocellular carcinoma cell lines causes epithelial–mesenchymal transition and increased invasiveness, suggesting that E-cadherin loss can be a causal factor of these phenotypes. Thus, E-cadherin plays critical roles in maintaining homeostasis and suppressing carcinogenesis in the liver.

liver progenitor cell | cholangiocellular carcinoma | mixed type tumor

E-cadherin is an important adhesion molecule, and not only establishes the core of the epithelial adherens junction with neighboring cells but also participates in intracellular signaling (1, 2). E-cadherin knockout mice die early during embryogenesis due to failed blastocyst and trophectoderm formation (3). Conditional knockout of E-cadherin in skin impairs an epidermal water-barrier function that leads to perinatal lethality (4). In addition, E-cadherin deletion in the differentiating alveolar epithelial cells of mammary gland results in an impaired differentiation program during lactation (5). Thus, although E-cadherin is a key regulator of embryonic development and tissue homeostasis, its role varies depending on the organ. Adult liver parenchyma consists of two types of hepatic epithelial cells, hepatocytes and biliary epithelial cells (BECs), and both cell types express E-cadherin localizing at the junctional complex (6). However, the precise functional role of E-cadherin in the liver is still unknown, especially in vivo.

Dysregulation of E-cadherin also contributes to cancer progression. In fact, mutation or decreased expression of E-cadherin is associated with malignant progression in various cancers, such as gastric, breast, and skin cancer (7). Also in human liver cancers, such as hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC), E-cadherin expression is decreased by 20–60% and is associated with higher histological grade, invasiveness, and poor prognosis (8–10). These findings suggest that E-cadherin may be a tumor suppressor in liver tumorigenesis. However, down-regulation of E-cadherin in liver cancer is caused by several mechanisms, including loss of heterozygosity, methylation of the E-cadherin promoter region, transcriptional repressors, and gene-silencing microRNAs (miRs) (8, 11–13). Transcriptional repressors such as Snail, Slug, and Twist, as well as miR-9, play an important role in induction of the epithelial–mesenchymal transition (EMT), which is a major cancer progression-mediating process. E-cadherin is a major target of these factors; however, they also control other EMT-inducing molecules involved in junctional complexes, intermediate filament networks, and the actin cytoskeleton (14). Therefore, it is unclear whether the loss of E-cadherin is a real culprit or a bystander in EMT induction and liver cancer progression. In addition, expression of E-cadherin can be increased during the early stages of HCC (11), and it has been suggested that preservation of E-cadherin expression may be beneficial for tumor growth, invasion, and metastasis (11, 15). Thus, here we characterize the role of E-cadherin in liver homeostasis and carcinogenesis in vivo using liver-specific E-cadherin knockout mice.

Results

Spontaneous Portal Inflammation and Periductal Fibrosis in *CDH1*$^{\Delta L}$ Mice. Liver-specific E-cadherin knockout mice ($CDH1^{\Delta L}$) were generated by crossing CDH1 flox/flox ($CDH1^{F/F}$) mice with

Significance

The precise roles of E-cadherin in the liver and liver carcinogenesis are still unknown. Here we show that mice lacking E-cadherin in the liver develop spontaneous periportal inflammation via an impaired intrahepatic biliary network, as well as periductal fibrosis, which resembles primary sclerosing cholangitis. Inducible gene knockout studies identified E-cadherin loss in biliary epithelial cells as a causal factor of cholangitis induction, and dysregulated E-cadherin expression was also seen in patients with primary sclerosing cholangitis. E-cadherin loss also significantly accelerates genetically and chemically engineered liver cancer through epithelial–mesenchymal transition, up-regulation of stem cell markers, and ERK activation. Thus, E-cadherin plays critical roles in maintaining homeostasis and suppressing carcinogenesis in the liver.

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albumin-Cre transgenic (*Alb-Cre*) mice. Immunohistochemistry (IHC) and immunofluorescence (IF) revealed that E-cadherin was expressed in the membrane of hepatocytes, especially in zone 1, and in the interlobular BECs in 1-mo-old $CDH1^{F/F}$ mice, whereas it was completely absent from both hepatocytes and interlobular BECs in $CDH1^{\Delta L}$ mice of the same age (Fig. 1*A* and Fig. S1*A*). On the other hand, E-cadherin expression was preserved in the large bile duct that is near the common bile duct (Fig. S1*B*). These results are consistent with recent reports of Cre expression in hepatocytes and interlobular BECs of *Alb-Cre* mice (16).

The histology of the liver appeared almost normal in 1-mo-old $CDH1^{\Delta L}$ mice (Fig. 1*B*). However, at 2 mo of age, $CDH1^{\Delta L}$ mice spontaneously developed periportal inflammation, followed by periductal fibrosis resembling primary sclerosing cholangitis (PSC) at 8 mo of age (Fig. 1 *B* and *C*). IHC for α-smooth muscle actin confirmed activation of fibroblasts in the periductal area (Fig. 1*D*). These histological changes were not seen in $CDH1^{F/F}$ mice. As is the case in cholestasis, serum levels of total bile acid and alkaline phosphatase (ALP) were significantly elevated in $CDH1^{\Delta L}$ mice compared with $CDH1^{F/F}$ mice (Fig. 1*E*). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining revealed that TUNEL-positive nonhepatocyte cells were noticeable in the periportal area in $CDH1^{\Delta L}$ mice (Fig. S1*C*). Although these cells were mostly $CD45^{+}$ leukocytes, they also contained K19-positive BECs, which were completely absent in $CDH1^{F/F}$ mice (Fig. S1 *D*–*F*). In comparison, there were no significant differences in serum alanine aminotransferase (ALT) and the number of TUNEL-positive hepatocytes between $CDH1^{F/F}$ mice and $CDH1^{\Delta L}$ mice other than a small increase of TUNEL-positive hepatocytes in 2-mo-old $CDH1^{\Delta L}$ mice (Fig. 1*E* and Fig. S1*G*). Hence, BEC injury could be one of the main factors contributing to development of periductal fibrosis.

Next, we postulated that cholestatic liver injury and periportal inflammation might be caused by disruption of the junction complex due to loss of E-cadherin. However, electron microscopy revealed no obvious morphological abnormalities in the adherens junctions, tight junctions, desmosomes, or bile canaliculi in 2-mo-old $CDH1^{\Delta L}$ mice (Fig. S1*H*). To functionally analyze the bile transport system, we injected fluorescent-labeled bile acid into the tail vein of 2-mo-old mice. Fifteen minutes after the injection, we could see clear canalicular staining in $CDH1^{F/F}$ mouse liver and smooth transport of bile acid into the bile duct lumen. In contrast, the canalicular staining pattern in $CDH1^{\Delta L}$ mice was very fuzzy, particularly in zone 1, and bile acid had not yet reached the bile duct lumen (Fig. 1*E*). These results suggest that the intrahepatic biliary network may be functionally impaired in $CDH1^{\Delta L}$ mice, which could lead to cholestatic liver injury and subsequent inflammation.

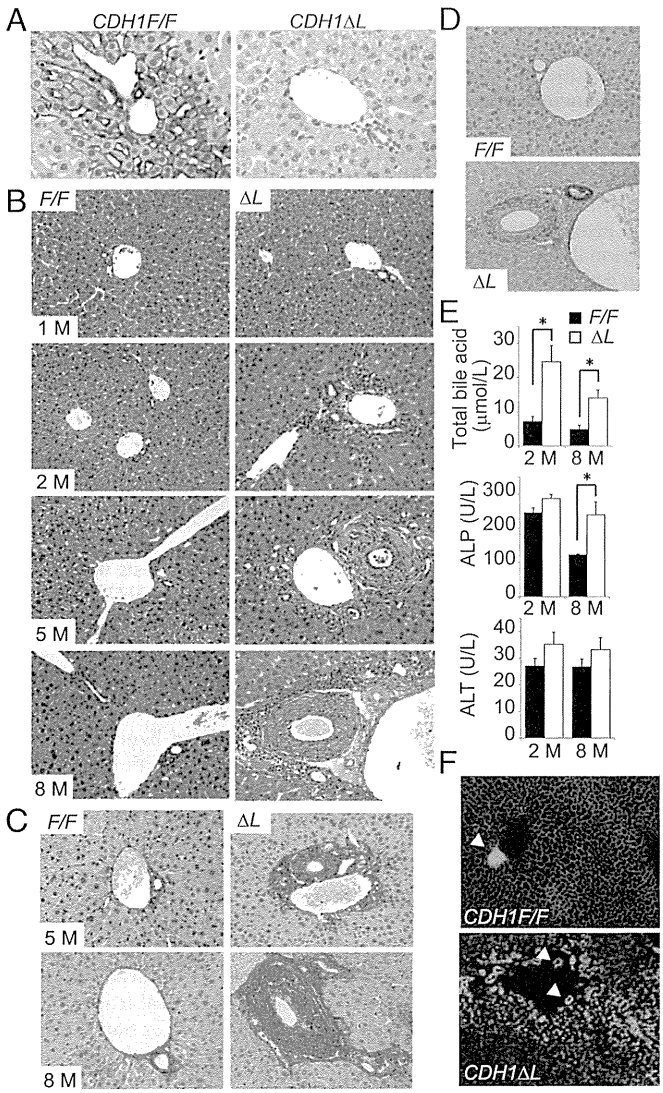

Fig. 1. Spontaneous development of portal inflammation and periductal fibrosis in $CDH1^{\Delta L}$ mice. (*A*) Analysis of E-cadherin expression by IHC of liver sections (×200) obtained from 1-mo-old $CDH1^{F/F}$ and $CDH1^{\Delta L}$ mice. (*B*) H&E staining of 1-, 2-, 5-, and 8-mo-old mice (representative images, ×200). (*C*) Sirius red staining in 5- and 8-mo-old mice (×200). (*D*) IHC of α-smooth muscle actin in 8-mo-old mice (×200). (*E*) Serum levels of total bile acid, ALP, and ALT in 2- and 8-mo-old mice. Results are means ± SEM (n = 5–7 per group; $*P < 0.05$). (*F*) Functional analysis of the bile transport system in 2-mo-old mice injected with fluorescent-labeled bile acid in the tail vein for 15 min followed by analysis of staining in the bile canaliculi (×200). Arrowheads indicate interlobular bile duct lumen (n = 3 per group).

Loss of E-Cadherin in BECs Rather than Hepatocytes Is a Causal Factor of Cholangitis Induction. To distinguish the function of E-cadherin in hepatocytes and BECs maintaining liver homeostasis, we generated two different models of E-cadherin deletion in the liver (Fig. 2*A*). First, to delete E-cadherin only in hepatocytes, we i.v. injected 5-wk-old $CDH1^{F/F}$ mice with adenovirus expressing Cre-recombinase (Ad-Cre) or control adenovirus (Ad-Cont) (17). We confirmed that this method induced Cre-loxP recombination in 73.0 ± 4.2% (mean ± SD) of hepatocytes but no recombination in BECs at 1 wk after injection using Rosa26-lox-stop-lox-YFP mice (Fig. S2*A*). Although E-cadherin expression was still reduced significantly in Ad-Cre–injected $CDH1^{F/F}$ mice at 8 wk after injection (Fig. S2 *B* and *C*), there was no apparent periportal inflammation (Fig. 2*B*). Next, to delete E-cadherin only in BECs, we crossed $CDH1^{F/F}$ mice with $K19^{CreERT}$ mice in which tamoxifen (TAM)-inducible Cre ERT was knocked into the endogenous K19 locus ($CDH1^{F/F}/K19^{CreERT}$) (18). According to the previous study, which showed relatively low efficacy of recombination in BECs, we injected TAM twice, at 5 and 9 wk after birth. One week after the first TAM injection into $CDH1^{F/F}/K19^{CreERT}$ mice, E-cadherin expression was deleted in 31.2 ± 7.2% of K19-positive BECs, whereas E-cadherin in the hepatocytes was well-preserved (Fig. S2*D*). Eight weeks after the first TAM injection, four of eight $CDH1^{F/F}/K19^{CreERT}$ mice revealed significant periportal inflammation as seen in $CDH1^{\Delta L}$ mice (Fig. 2*C*). Although the rate of E-cadherin loss in BECs varied widely in TAM-injected $CDH1^{F/F}/K19^{CreERT}$ mice at this point (25.5 ± 17.1%), bile ducts with strong inflammation tended to show high rates of E-cadherin deletion (Fig. 2*D* and Fig. S2*E*). Thus, loss of E-cadherin in BECs rather than hepatocytes is a causal factor of periportal inflammation. Furthermore, in human liver samples, a clear membranous pattern of E-cadherin expression in the epithelial cells of medium-size bile ducts was seen in normal liver, whereas it mostly disappeared, with only fragmented cytoplasmic expression, in four of seven PSC samples (Fig. 2*E*). In contrast, E-cadherin