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ARTICLE

Metformin prevents liver tumourigenesis by attenuating fibrosis in a transgenic mouse model of hepatocellular carcinoma

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Abstract

Metformin is a hypoglycaemic agent used to treat type 2 diabetes mellitus (DM2) patients, with a broad safety profile. Since previous epidemiological studies had shown that the incidence of hepatocellular carcinoma (HCC) decreased significantly in metformin treated DM2 patients, we hypothesised that intervention with metformin could reduce the risk of neoplastic transformation of hepatocytes. HCC is the most common primary liver malignancy and it generally originates in a background of liver fibrosis and cirrhosis. In the present study, we took advantage of a transgenic mouse (TG221) characterized by microRNA-221 overexpression, with cirrhotic liver background induced by chronic administration of carbon tetrachloride (CCl4). This mouse model develops fibrosis, cirrhosis and liver tumours that become visible in 100% of mice at 5–6 months of age. Our results demonstrated that metformin intervention improves liver function, inhibits hepatic stellate cell (HSC) activation, reduces liver fibrosis, depletes lipid accumulation in hepatocytes, halts progression to decompensated cirrhosis and abrogates development HCC in CCl4 challenged transgenic mouse model. The study establishes the rationale for investigating metformin in cirrhotic patients regardless of concomitant DM2 status.

Introduction

Liver cancer is the second leading cause of cancer related death worldwide, with about 841,000 new cases annually [1]. Therapeutic options are limited, and prognosis is generally poor, with curative approaches available only for early stages of disease. Hepatocellular carcinoma (HCC) arises from hepatocytes and is the most common primary liver cancer. About 80 to 90% of all HCCs occurs within a background of chronic liver disease and cirrhosis, which represents the most important risk factor for HCC [2]. The extended time of progression from liver fibrosis to malignancy make individuals at risk for HCC identifiable and candidates for chemoprevention strategies to delay or stop the natural course of chronic liver disease and curtail HCC incidence and mortality [3].

Hepatitis B vaccination programs or anti-hepatitis C therapeutics are examples of approaches that not only protect from viral infection, but also reduce the incidence of virus-associated HCC [4, 5]. However, non-viral causes namely alcoholic or non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) account for nearly 46% liver cancer deaths [6]. Additionally, increasing epidemic of obesity and type 2 diabetes mellitus (DM2), both important risk factors of NAFLD and NASH, might emerge as dominant risk factors for HCC incidence in future [7, 8]. Hence, prevention programs can have an important impact in decreasing HCC occurrence.

Metformin is a first-line dimethylbiguanide hypoglycaemic agent used to treat DM2 patients, with a broad safety profile. Abundant epidemiological data have indicated that incidence of several cancers, including HCC decreased

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significantly in DM2 patients on metformin treatment compared to dietary restrictions alone, insulin or sulfonylureas [9–12]. Several mechanisms have been proposed for metformin action. They include metabolic reprogramming of cancer cells by activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) [13, 14], altering vascular and immune tumour microenvironment [15, 16] and suppressing stemness of cancer cells [17, 18].

We hypothesized that intervention with metformin in non-diabetic liver fibrosis/cirrhosis setting could reduce tumour formation. Despite numerous pharmaco-epidemiological data on cancer preventive effects of metformin in DM2, impact of metformin on HCC prevention in non-diabetic liver disease is largely unexplored. A meta-analysis of anti-tumour effects of metformin in HCC animal models concluded that pre-clinical studies lacked sufficient indication of stage at which metformin use is beneficial [19]. This is at least in part due to a dearth for animal models that uniformly manifest HCC with a background of cirrhosis, typically seen in human disease. In the present study, hepatoprotective effect of metformin was investigated in transgenic mice that develop liver tumours in a cirrhotic liver background.

Materials and methods

CCl4 induced HCC mouse model with cirrhosis background and metformin intervention

Male miR-221 transgenic (TG221) strain with B6D2F2 background mice (4–6 weeks of age) were administered 150 µl of olive oil (Arm 1) or 20% v/v CCl4 in olive oil (Arm 2) by oral gavage (p.o.) three times a week for a duration of 14 weeks. Metformin intervention (Arm 3) was started after 3 weeks of initiating CCl4 challenge at 300 mg/kg body weight (BW) daily p.o., dissolved in distilled water. Dosage of metformin used in present study was calculated using Reagan-Shaw method [20]. Briefly mouse equivalent dose (mg/kg) = human dose (mg/kg) × human (k_m)/mouse (k_m). Where k_m factor, unique to each species is a constant used to normalize dosage based on body surface area. For a 60 kg human adult k_m equates to 37 and a 20 g mouse k_m equals 3. Metformin daily dosage in humans range from 1000 mg to 2500 mg, usually prescribed twice daily. Therefore, a dose of 1500 mg per day in human adults translated to approximately 300 mg/kg per day mouse dosage [15]. Mice were randomly assigned to the different experimental arms. Ellipsoid volume of surface nodules were measured ex-vivo as $V = (\pi/6) \times$ (long axis) × (short axis) [21]. Mice were maintained in vented cabinets at 24 °C with a 12-h light-dark cycle. Food and water were provided ad libitum. All animal procedures were performed according to the guidelines of the Italian Ministry of Health Public Health Service Policy on Care and Use of laboratory animals, and in accordance with a protocol approved by the Institutional Animal Care Committee of University of Ferrara and Italian Ministry of Health.

Transaminase assays

At time points of 0, 3, 6, 9, 14 weeks blood sampling of 20 µl was done by tail vein nick and post 24 weeks by cardiac puncture after mice were euthanized by isoflurane inhalation anaesthesia and subsequent cervical dislocation. Serum was collected from blood samples of corresponding experimental groups and AST and ALT levels assayed with respective colorimetric kits (Sigma; MAK055/MAK052) according to manufacturer's instructions and absorbance read on Tecan Infinite F200 Pro plate reader (TECAN, Switzerland).

Ultrasonography

Liver ultrasonography (Philips iU22 with a linear transducer) surveillance was performed at fortnightly intervals after 14 weeks to monitor growth of liver nodules. Mice were anesthetized with intraperitoneal (i.p.) cocktail of ketamine (90 mg/k BW) and xylazine (9 mg/kg BW) in 0.9% sodium chloride solution and were always placed on temperature-controlled heating pads during the entire imaging procedure. DICOM files were analyzed using an open source medical image viewer (Horos project v3.1.2).

Cell lines and reagents

Human HCC cell line HepG2 (ATCC HB-8065, Rockville, MD, USA and authenticated by the provider by cytogenetic analysis) were cultured as monolayers in IMDM media (Sigma; I6529) supplemented with 10% fetal bovine serum (FBS, Sigma; F7524), 0.1% gentamicin (Gibco; 15750-037). Human hepatics stellate cell line LX-2 (generous gift from Vienna Hepatic Experimental Hemodynamic-HEPEX Lab at the Medical University of Vienna) were cultured as monolayer in DMEM media (Sigma; D5796) supplemented with 5% FBS, 0.1% gentamicin. All cell lines were maintained in a 37 °C humidified incubator containing 5% CO_2 and tested free of mycoplasma contamination (MycoAlert Mycoplasma Detection kit, cat#LT07-418, Lonza). Metformin (Sigma; D150959) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mol/L, stored at −20 °C and subsequently diluted to appropriate concentrations in complete media without antibiotics for each assay. All cell culture experiments were carried out within eight passages after being thawed, performed in triplicates and repeated twice.

Western blot

Snap frozen tissues were homogenized in RIPA buffer (Sigma R0278) with protease/phosphatase inhibitors (complete ULTRA/PhosSTOP tablets, Roche) and sodium orthovanadate using a tissue homogenizer. Lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. Protein concentration of the supernatant was measured by modified Lowry protein assay before boiling in 4× Laemmli buffer. Approximately 10 µg of protein from lysates were loaded on precast PAGE, separated by electrophoresis and transferred to pvdf membranes with Trans-Blot Turbo system (Bio-Rad). Non-specific binding was blocked by 5% non-fat milk for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies. Primary antibodies used: LKB1 (Cat#3047), AMPKα (Cat#2532), pAMPKα Thr-172 (Cat#2535), AKT (Cat#4691), pACC Ser-79 (Cat#3661), ACC (Cat#3662), S6 (Cat#2217), p4E-BP1 (cat#2855), 4E-BP1 (Cat#9644), PARP (Cat#9542), cleaved-PARP (Cat#9544) at 1:1000 dilution and pAKT Ser473 (Cat#4060), pS6 Ser-235/236 (Cat#4858) at 1:2000 dilution. All antibodies listed above were obtained from Cell Signalling Technologies (Danvers, MA, USA). Other antibodies used were: α-SMA (1:500 Cat#A5228, Sigma) and GAPDH as loading control (1:5000 Cat# TA890003, Origene). Following day, after a brief wash, membranes were incubated with anti-rabbit HRP (Cell Signaling 1:1000) or anti-mouse HRP (Cell Signaling 1:5000) secondary antibodies. Membranes were developed with Clarity western ECL (Bio-Rad) or high-sensitivity Westar Supernova Blotting Substrate (Cyanagen, Italy) and chemiluminescence visualized on Chemidoc XRS+ imaging system (Bio-Rad).

Histology and immunocytochemistry

Formalin-fixed paraffin embedded (FFPE) samples were sectioned into 5 µm-thick sections and stained with hematoxylin-eosin (H&E) and Masson's trichrome according to standard procedures. All slides were reviewed by the same pathologist. Formalin-fixed samples were further processed with 30% sucrose and embedded in optimal cutting temperature (OCT) compound and frozen to −80 °C. OCT embedded samples were sectioned into 10 µm-thick sections and stained with Oil Red O (ORO) staining according to standard procedures. Lipid droplets were morphometrically quantified on ORO stained sections with image processing software (ImageJ, NIH) as described previously [22]. In total, 10 fields of visions were analyzed for each section and lipid droplet size and number were averaged for comparison. HepG2 cells cultured with or without metformin were stained with ORO and images were captured. Amount of lipid droplets were quantified semi-quantitatively by extracting ORO from cells in 100% propanol and absorbance measured at OD450nm with Tecan Infinite F200 Pro plate reader (TECAN, Switzerland). Fixed LX-2 cells cultured on polylysine coated round coverslips were incubated with α-SMA antibody (1:100, Cat#A5228, Sigma), followed by incubation with Alex Fluor® 488 goat-anti mouse (1:1000, Cat#A11001, Thermo Fischer scientific) for 1 h at room temperature. Cells were then mounted with Vectashield mounting medium with DAPI, observed with a Nikon Eclipse TE2000-E confocal microscope (Nikon, Florence, Italy) and images acquired by Nikon DXM1200F digital camera.

Gene expression microarray analysis

Gene expression profiling was done with an Agilent whole mouse gene expression 8 × 60 K microarray (Cat# G4852A, Agilent Technologies). One-colour microarray-based gene expression was analyzed according to standard operating procedures from Agilent Technologies. Briefly, total RNA from snap frozen samples was extracted using Trizol Reagent (FS-881, FMB, Trevose, USA) according to manufacturer's instructions. Quantity and quality of RNA was assessed with RNA-600 nanochip (Agilent Technologies) on Agilent 2100 Bioanalyzer. Samples with RNA integrity number above 8 were utilized for microarray. Total RNA of 100 ng from each sample was used to synthesize cyanine 3-CTP (Perkin-Elmer Life Sciences, Boston, USA) labelled cRNA with Low RNA Input Linear Amplification kit (Agilent Technologies). Labelled RNA was hybridized at 65 °C for 17 h at 10 rpm in an incubator. Images of slides were captured by the Agilent scanner and raw microarray data was obtained by accompanying Agilent Feature Extraction Software (v10.5). Quantile normalization of raw microarray expression and downstream analysis was performed with Qlucore omics explorer (v3.4, Qlucore AB, Sweden). Differentially expressed genes in two group comparison were sorted based on analysis of variance, *F*-test and <0.01 false discovery rate (FDR-q). Heat maps were generated based on hierarchical clustering of samples and genes. Genes that were differentially expressed with fold change >1.5 and FDR <0.01 between two groups (CCl4+metformin vs CCl4 livers) on an unpaired t-test were considered for Gene Set Enrichment Analysis (GSEA). Differentially expressed genes were summarized into mouse Entrez gene IDs and mapped to human orthologs using mapping reports from Mouse Genome Informatics database (Jackson laboratories- www.informatics.jax.org). Differential genes between phenotypes were ranked according to t-statistics. Metformin mediated up-regulated genes were given a positive score and corresponding down-regulated genes a negative score compared to CCl4 only livers. Pre-ranked GSEA was applied with GSEA

3.0 software [23, 24]. The curated canonical pathways from MSigDB (Molecular Signature Database - Hallmark, KEGG and Reactome) were used. Statistical significance of normalized enrichment score was estimated using phenotype-based permutation (1000) testing and FDR <0.25. Raw data from microarrays are available at Gene Expression Omnibus (GEO accession number: GSE131175). Predicted miR-221 mouse gene targets were retrieved from miRDB excluding targets with >70 prediction score (244 genes, Supplementary excel file) and commonality was compared with metformin mediated dysregulated proteins elucidated by immunoblotting [25].

Statistical analyses

In mouse studies, no animals were excluded from the analysis. Investigators were not blinded after simple randomization method used to assign mice to different experimental arms. Sample size for animal experiments were calculated to be seven mice for each CCl4 and metformin + CCl4 groups at significance level alpha of 5%, a priori power 80% and estimated effect side (*d*) 1.5, using G*Power software [26]. An unpaired *t* test (two-tailed with unequal variance) was used to compare differences between two groups throughout the study and significance with a threshold of *P*-value <0.05 was considered. Variances between groups were compared by *F*-test. Summary data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using data analysis software Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

CCl4 induced cirrhosis and HCC in the TG221 mouse model

We utilized a previously established transgenic mouse strain (TG221), which is predisposed to the development of liver tumours [27]. Recurrent liver injury was induced in 4 to 6 weeks old male mice by oral gavage of 150 µl 20% CCl4 v/v in olive oil, three times a week for the duration of 14 weeks. Control TG221 mice were administered 150 µl of olive oil only, at the same time points (Fig. 1a). Serum levels of liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) significantly increased with time, denoting liver damage in CCl4 challenged mice when compared to olive oil treated mice (Fig. 1b). Notably, serum transaminase levels remained high also after discontinuation of CCl4, thus indicating that an irreversible stage of liver damage was reached.

Mice were sacrificed 3, 6, 9 and 14 weeks after initiating CCl4 or olive oil gavage ($n = 2$ for each time point) and liver tissues were harvested. Macroscopically, livers showed signs of the CCl4-induced damage, characterized by a yellowish pale appearance rather than dark red, a harder than normal consistency, an irregular surface with nodules of different sizes (Fig. 1c). Trichrome staining revealed a sequential progression of liver fibrosis with sustained CCl4 challenge. Thin septal fibrosis was evident at 3 weeks, progressing to bridging fibrosis by 6 and 9 weeks but still maintaining liver architecture. However, 14 weeks of CCl4 challenge led to progression of fibrosis, distortion of liver architecture, nodule formation and ascites typical of cirrhosis (Fig. 1d, Supplementary Fig. 1a).

Following cessation of Olive oil (Arm1, $n = 7$) or CCl4 gavage (Arm2, $n = 7$), mice were monitored by abdominal ultrasonography (USG) at bi-weekly intervals for signs of tumour development. While small liver nodules could be identified at week 14, frank liver masses were detected by USG at 24 weeks only in Arm2 mice (Supplementary Fig. 1a, b).

At the experimental end-point (24 weeks), explorative laparotomy revealed hepatomegaly and multiple surface nodules in 100% of CCl4 challenged mice. Olive oil treated mice did not present with hepatomegaly or surface nodules in the liver (Supplementary Fig. 1c, d). Histological assessment of livers and corresponding nodules revealed different phases of tumourigenesis from dysplastic focal nodular hyperplasia (FNH)-like, to frank HCC with abundant steatosis (Supplementary Table 1). In accordance with gross pathology, histopathology of livers from olive oil treated mice did not reveal any evident liver lesions.

Metformin intervention abrogates fibrosis and steatosis in CCl4 challenged TG221 mice

In patients, progression from asymptomatic liver fibrosis to decompensated cirrhosis takes years. Furthermore, once end-stage liver disease is reached, liver transplantation represents the only option to improve quality of life in these patients [28]. Therefore, implementation of early intervention strategies are aimed at preventing deterioration of fibrotic disease [29]. Since, in the TG221 mouse model, CCl4 induced fibrosis as early as 3 weeks, we hypothesized that chemoprevention strategies at this stage could possibly prevent the progression of disease. TG221 mice ($n = 7$) were treated daily by oral gavage with metformin at a dosage of 300 mg/kg, starting at 3 weeks post induction of CCl4-challenge (Fig. 2a). Dosage of metformin used in present study was calculated according to the Reagan-Shaw method [20] to approximate human daily dosage of 1500 mg, typically taken by patients affected by type 2 DM. These mice were administered CCl4 up to 14 weeks, like in Arm2 control, and daily metformin treatment was continued until the experimental end-point at 24 weeks. Of note,

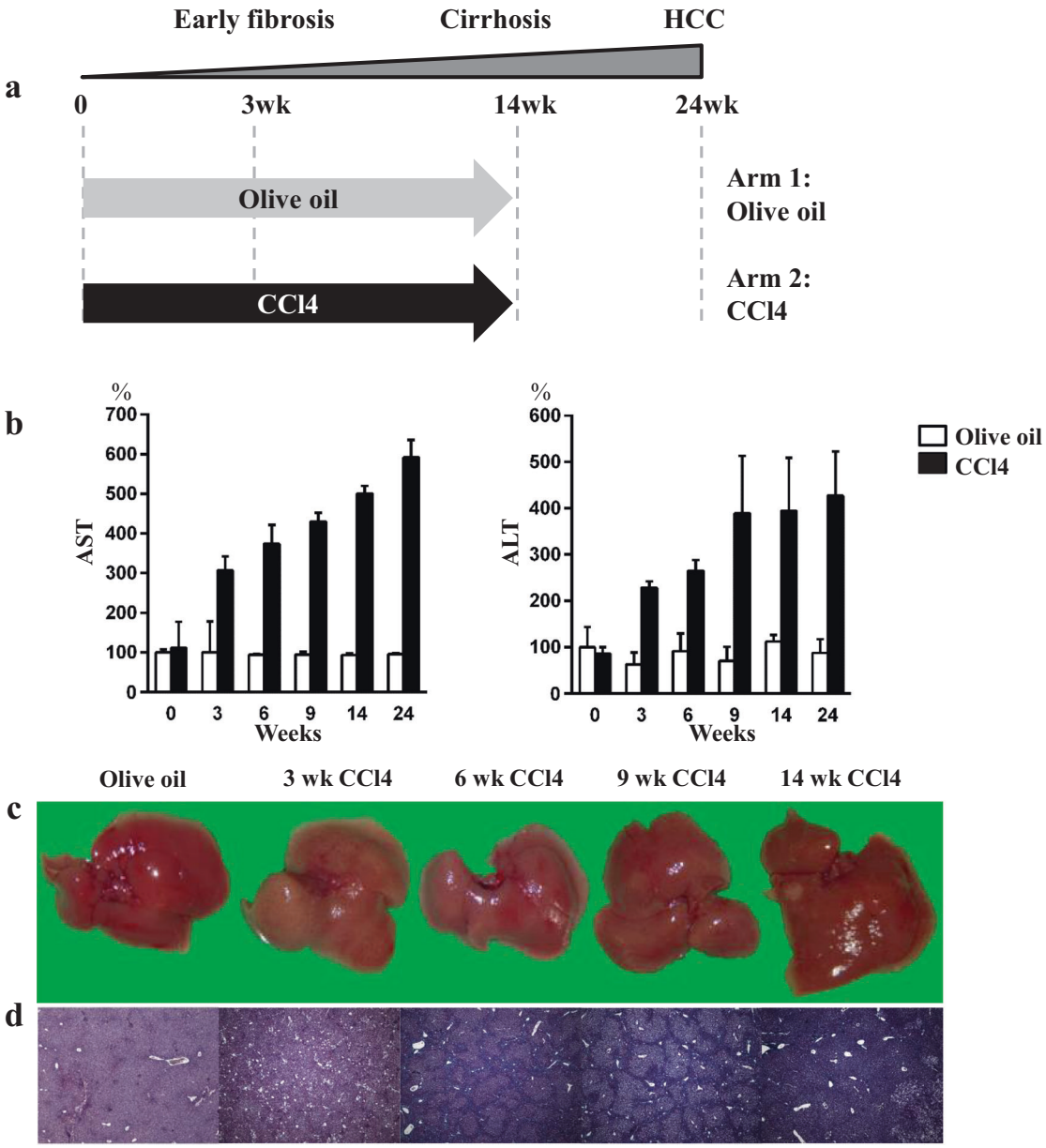

Fig. 1 CCl4 administration induces chronic liver damage and fibrosis in the liver of mice. **a** Experimental design: 4 to 6 weeks old mice were administered olive oil (Arm1) or 20% CCl4 in olive oil (Arm2) per os (p.o.) for 14 weeks. Experimental end-point was at 24 weeks after initiation of CCL4 challenge. **b** Liver damage was detected by the increase of liver Aspartate transaminase (AST) and Alanine transaminase (ALT) enzyme levels. AST-ALT levels are depicted as percentage change between Olive oil and CCl4 experimental arms. **c** Images of mouse livers at the indicated time of sacrifice. **d** Trichrome staining of FFPE livers at corresponding same time points and treatments as of (**c**) (×40 magnification)

treatment with metformin significantly reduced serum levels of AST and ALT (Fig. 2b). Three-hours post final metformin gavage, mice were sacrificed, and livers collected.

Metformin intervention significantly decreased CCl4 induced liver fibrosis as evidenced by collagen content, immunoblotting for α-SMA and gene expression analysis (Fig. 3a–c). The reduction of *Col1a1*, *Col3a1*, *Col4a1* expression and α-SMA protein levels in livers indicates an inhibitory effect on hepatic stellate cells (HSCs) activation by metformin. We confirmed that metformin abrogated the expression of α-SMA in the human HSCs LX-2 cells in vitro (Supplementary Fig. 2a, b).

Microvesicular steatosis is another feature of cirrhosis where excess lipid droplets are observed in hepatocytes [30, 31]. Metformin significantly decreased accumulation of lipid droplets in livers when compared to untreated CCl4 challenged mice (Fig. 4a, b). One of the well-studied mechanism of actions of metformin is its ability to activate

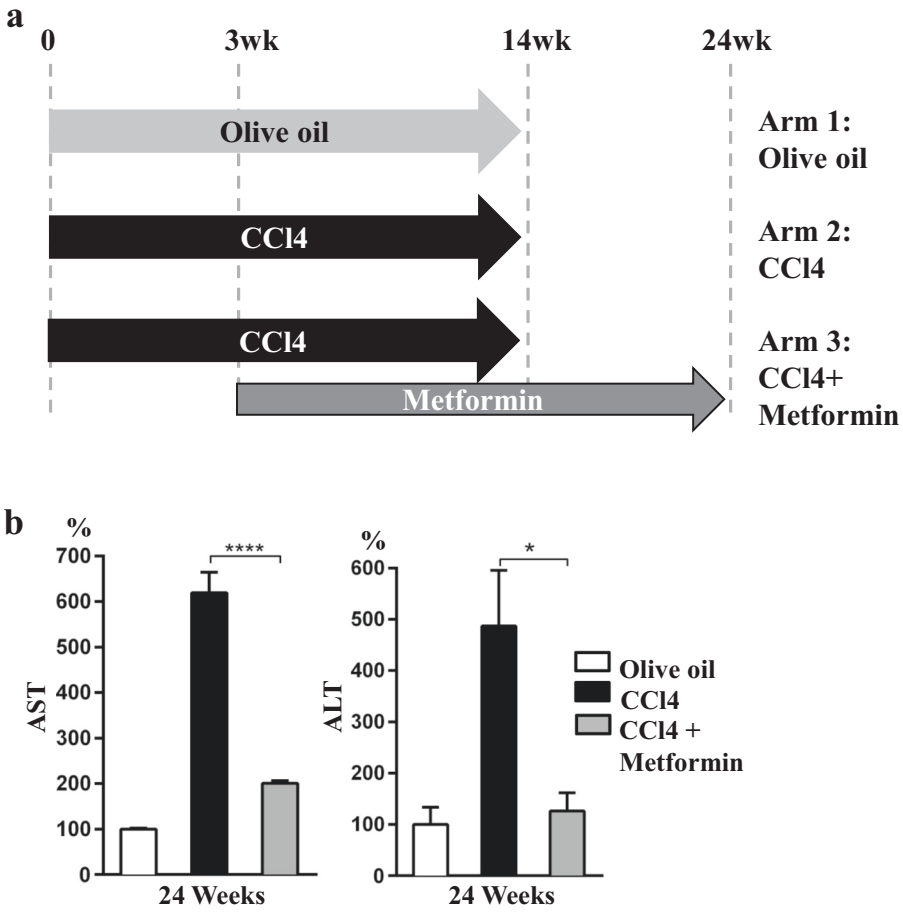

Fig. 2 Experimental design of metformin treatments. **a** Mice were administered olive oil (Arm1) or 20% CCl4 in olive oil (Arm2 and Arm3) per os (p.o.) for 14 weeks. Additionally, mice in Arm3 were treated with Metformin at 300 mg/kg dosage p.o., starting at 3weeks post initiation of CCl4 challenge, until experimental end point at 24 weeks. ($n = 7$ in each experimental arms). **b** Metformin intervention improved serum levels of the liver enzymes AST and ALT. ****$P < 0.0001$ *$P < 0.05$. AST-ALT levels are depicted as percentage change between experimental arms at 24 weeks time-point

AMPK [32]. AMPK has been attributed to be a master regulator of cellular metabolism and energy homoeostasis [33]. Here, we confirmed by immunoblotting, that metformin increased the levels of total LKB1 leading to increased phosphorylation of AMPK at Thr-172. As a consequence of activation of AMPK, downstream inhibitory phosphorylation of ACC at Ser-79 was also uncovered (Fig. 4c). The enzyme ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis [34]. Abrogation of ACC activity might account for metformin mediated reduction of de novo lipogenesis in hepatocytes. Further, in vitro metformin significantly reduced intracellular lipid droplets in HepG2 cells in a dose and time dependent manner (Supplementary Fig. 3).

Metformin intervention prevents HCC in CCl4 challenged TG221 mice

Metformin intervention at an early fibrosis stage dramatically abrogated formation of tumour nodules (Fig. 5). Histopathology of livers did not reveal any detectable in situ nodules in metformin treated mice, whereas in CCl4-only mice, abundant nodules varying from FNH-like to HCC were noticed (Supplementary Table 1, Supplementary Fig. 4).

Data on fibrosis and steatosis suggest that the change in the microenvironment could prevent tumour appearance. Additionally, GSEA performed on microarray data from livers of untreated and metformin treated CCl4 exposed livers suggested direct effects on cancer-associated pathways. Hallmark gene sets enriched significantly for mTORC1, KRAS and PI3K/AKT/mTOR signaling pathways (Supplementary Fig. 5), with metformin negatively regulating these pathways. Confirming these indications, we observed that metformin inhibited AKT phosphorylation in liver tissue along with downstream key effectors S6 and 4E-BP1 (Fig. 6). Albeit, metformin had no apparent effect on total or phosphorylated status of mTOR (data not shown). Since PI3K/AKT pathway is a key regulator of cell survival [35], metformin-mediated inhibition of AKT resulted in a higher level of apoptosis as demonstrated by the increased cleavage of PARP (Fig. 6, Supplementary Fig. 6). None of the proteins dysregulated through metformin treatment were direct predicted miR-221 targets.

Metabolism and bioactivation of CCl4 in liver are primarily mediated by cytochrome P450 2E1 (CYP2E1)

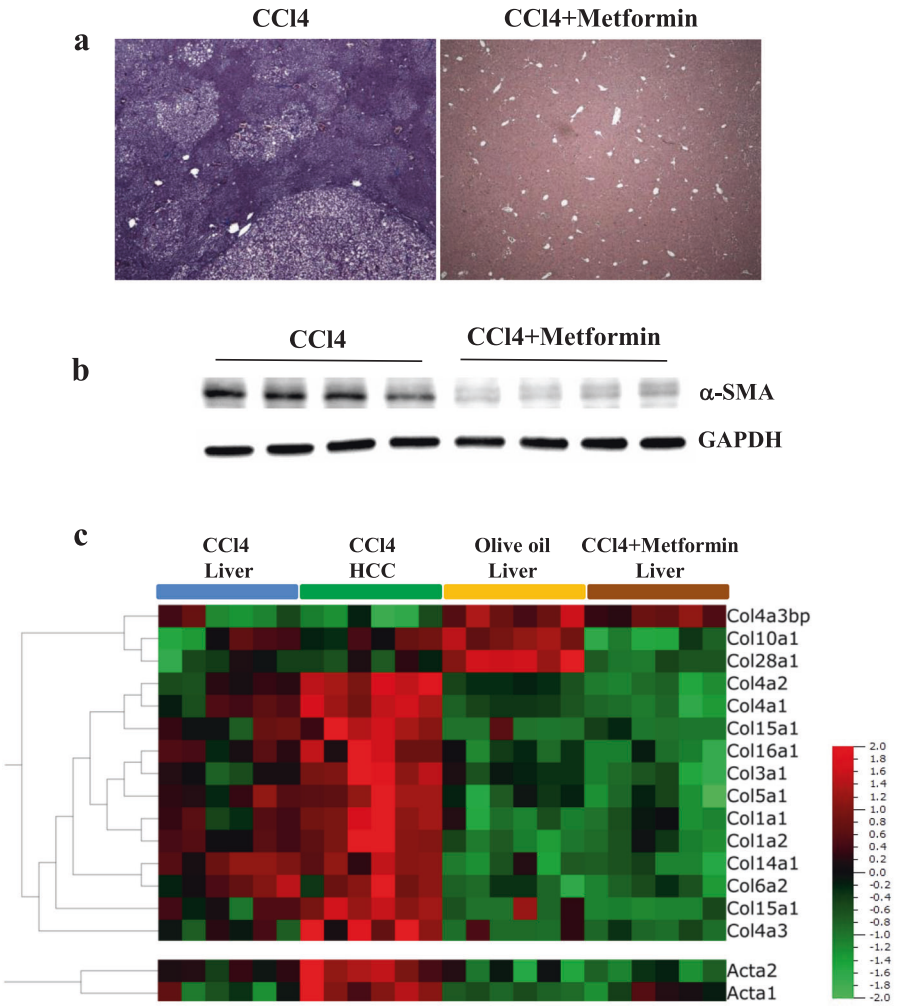

Fig. 3 Metformin intervention reduces fibrosis in CCl4-challenged TG221 mice. **a** Representative trichrome staining of FFPE livers at 24 weeks without and with metformin intervention. **b** Western blot analysis of a-SMA protein in livers without and with metformin intervention. **c** Heat map for the expression of several collagen and α-SMA genes (*Acta2*) in CCl4 only livers, matched liver nodules, olive oil livers and CCl4 +metformin treated livers. Intense red means the highest expression; intense green the lowest. Uncropped images of western blots are shown in Supplementary Fig. 6

enzyme [36]. Thus, metformin could negatively regulate CYP2E1 expression and abrogate CCl4-mediated liver injury. However, we observed that gene expression of *Cyp2e1* in olive oil, CCl4 and metformin+CCl4 treated livers was not significantly dysregulated (Supplementary Table 2). Furthermore, GSEA was not significant for genes involved in regulation of transcription factor activity or several other cytochrome P450 enzymes involved in metabolism of xenobiotics or drugs were not enriched upon metformin intervention (Supplementary Table 3).

Discussion

By acting on aetiologic viral factors, like HBV or HCV, it has been possible to reduce the risk of virus-associated HCC. Either Hepatitis B immunization programs or anti-HCV direct-acting treatments have not only been beneficial to virus control, but also in reducing viral-associated HCC incidence [4, 37]. However, chemoprevention strategies in non-viral related HCC remain a significant unmet medical need. Although liver is the primary site of metformin function, to the best of our knowledge, hepatoprotective effects of early metformin intervention has been previously reported only once in a carcinogen-induced rat model of HCC [17]. The effect of metformin has also been investigated in patients with advanced HCC under sorafenib therapy. In this setting, metformin was not found to be effective, even possibly responsible for an increased tumour aggressiveness and a reduction of overall survival [38].

Several challenges are present in the preclinical development of secondary chemoprevention. Firstly, the difficulty of developing reliable mouse models capable of simulating the progressive evolution of liver disease from fibrosis to HCC has hitherto prevented testing experimental prevention approaches. To this end, CCl4 induced chronic liver injury in TG221 mouse model could reproduce all the mentioned phases and was crucial to perform prevention studies reported in this present study. Secondly, since pharmacological agents for early intervention are required to be administered for long periods of time, dictated by extended time to cancer progression, these agents should

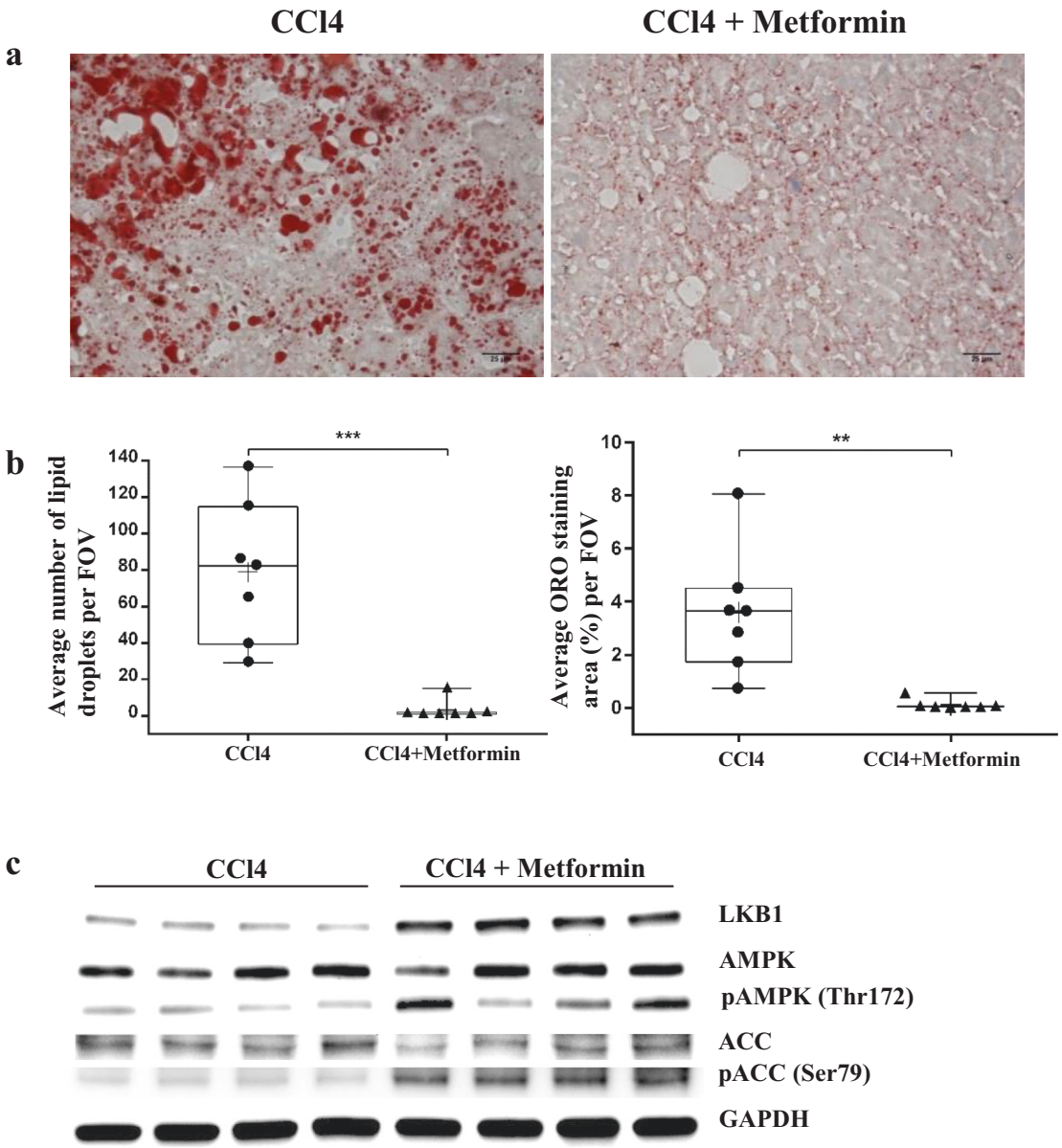

Fig. 4 Metformin intervention reduces steatosis in CCl4-challenged TG221 mice. **a** Oil Red O (ORO) staining of sections from formalin fixed, OCT embedded and frozen livers at 24 weeks without and with metformin intervention. **b** Lipid droplets were morphometrically quantified after ORO staining for average number and percentage staining area per field of vision in CCl4 and CCl4+metformin mice. $^{***}P < 0.001$ $^{**}P < 0.01$. Each data point represents a single mouse. **c** Western blot analyses show that metformin induces LKB1 mediated AMPK activation and leads to the downstream inhibitory phosphorylation of ACC at Ser-79, a rate-limiting step in fatty acid synthesis. Uncropped images of western blots are shown in Supplementary Fig. 6

preferably be orally available and with minimal or no potential toxicity. Our results show that early intervention with metformin was effective in preventing HCC occurrence in the TG221-CCl4 mouse model. Oral administration of 300 mg/kg metformin, which approximately translates to standard 1500 mg/day taken by DM2patients, abrogated formation of liver tumours in mice, thereby suggesting that secondary chemoprevention of HCC could be achieved in patients with early signs of fibrosis at standard prescribed daily metformin dose.

Tumour initiation and progression is predominantly driven by conducive tissue environment that facilitates epithelial-mesenchymal transition (EMT), dysregulated metabolism and sustained proliferative signaling [39, 40]. Cirrhosis microenvironment comprises all these features and represents the most important risk factor for HCC development.

Our results indicate that metformin acts primarily to normalize fibrotic and steatotic microenvironment typical of cirrhosis. In fibrotic liver, activated HSCs produce various

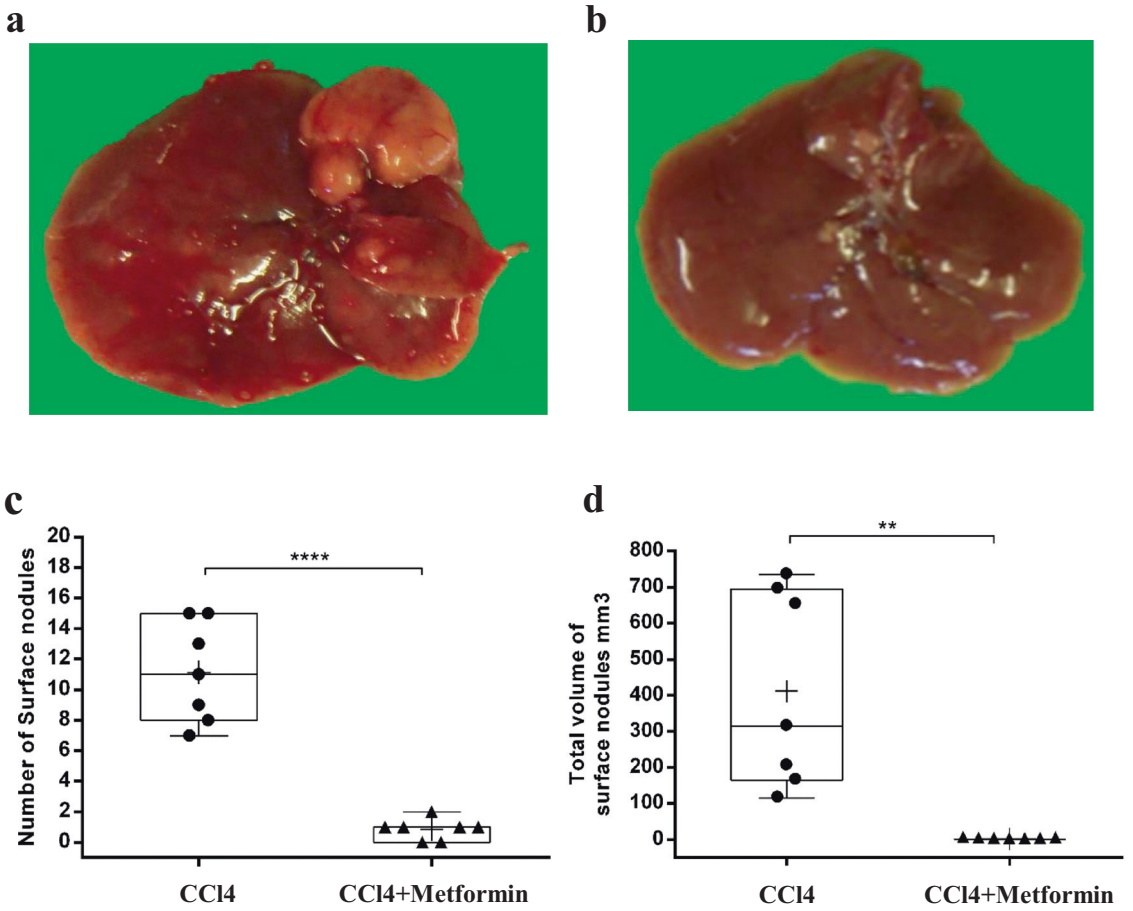

Fig. 5 Metformin intervention prevents the appearance of tumour nodules in CCl4-challenged TG221 mice. **a** Representative liver from a CCl4-challenged mouse control. **b** Representative liver from a mouse treated with CCl4 and metformin. **c**, **d** Surface nodules: number and volume in CCl4 mice in comparison with mice that received CCl4 +metformin. The latter did not develop any tumour nodules. $****P < 0.0001$ $**P < 0.01$ Each data point represents a single mouse

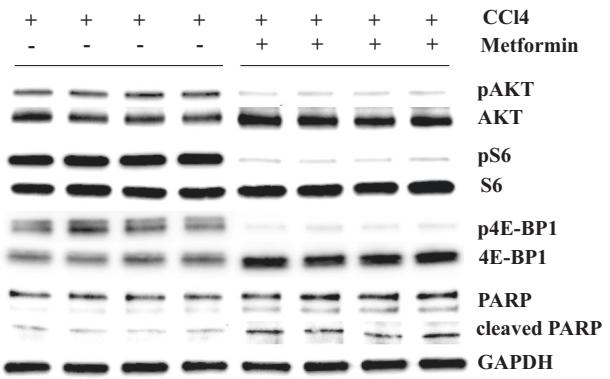

Fig. 6 Metformin intervention modulates the AKT pathway and apoptosis in liver tissue of CCl4-challenged TG221 mice. Western blot analyses of AKT, S6, 4E-BP1 and PARP in liver tissue from CCl4 and metformin treated mice. GAPDH was used an internal loading control. Uncropped images of western blots are shown in Supplementary Fig. 6

collagens and contribute to EMT [41, 42]. Here, we show that metformin inhibited the activation of HSCs as evidenced by reduced α-SMA and collagen (*Col1a1, Col4a1, Col4a2*) expression levels, thereby reducing liver fibrosis. Additionally, GSEA revealed negative regulation of set of hallmark EMT genes (NES = −2.40, FDR = 0.002) by metformin, which supports the inhibition of EMT process. Furthermore, metformin intervention resulted in LKB1 mediated AMPK activation, which is considered a key regulator of metabolic and energy homoeostasis and downstream inhibition of ACC [34, 43]. This resulted in significant reduction of microvesicular steatosis in livers. Earlier in vivo studies reported that loss of lipid droplets in HSCs in chronically injured livers might suppress tumourigenesis [44].

GSEA provided additional valuable insights into effects of metformin on cellular proliferation/survival signalling pathways, most importantly PI3K/AKT/mTOR pathway. Although we did not detect any effect of metformin on mTOR, suppression of AKT and its downstream effectors explains the pro-apoptotic liver environment which negatively influenced liver tumourigenesis. Indeed, liver injury induced by CCl4 resulted in liver enlargement and fibrosis. However, the apoptotic effect promoted by metformin

suggests that cell death could possibly antagonize liver proliferative reaction to chronic injury. Previous studies in carcinogen-induced and spontaneous mouse models have reported that targeting AKT or metformin mediated AKT suppression, has been beneficial in preventing lung tumours [45–47].

Our findings carry the promise for a medical application. While a number of studies established that metformin reduces the risk of developing HCC in type 2 DM patients [48–54] (Supplementary Table 4), our results indicate that its prophylactic activity can be expanded to patients with liver fibrosis at risk of developing cirrhosis and HCC, regardless of concomitant type 2 DM. The translational value is high, as it involves a potentially large number of individuals. According to epidemiological studies in US and Europe, prevalence of cirrhosis was estimated at about 0.3% of population [55, 56], probably an underestimate considering that early stages of cirrhosis are asymptomatic. In addition to reducing the risk of HCC occurrence, our results establish that the use of metformin in early stages of liver fibrosis can effectively ameliorate the underlying liver disease despite continued exposure to aetiological risk factors, suggesting that this approach could equip clinicians for a better management of chronic liver disease as well as prevention of HCC.

In conclusion, metformin intervention resulted in improved liver function, reduced fibrosis, decreased dependence on lipids as primary source of energy and most importantly abrogated the appearance of liver tumours. Low cost, long-term tolerability and safety of metformin provide a strong rationale for chemoprevention in liver fibrosis patients who are at high risk of developing liver cancer.

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Compliance with ethical standards

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

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