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Research article

Metformin dampens the progression of cholangiofibrosis induced by thioacetamide using deep learning

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ABSTRACT

Purpose: The consistent use of metformin has been linked to a reduced incidence of neoplastic diseases among diabetic populations. As a preventive intervention, metformin may offer a more favorable risk-benefit profile. Here, we explored the efficacy of metformin in the primary prevention of cholangiofibrosis, which can precede the carcinogen-induced development of cholangiocarcinoma (CCA). Our objective was to assess the potential of metformin to act as an intervention prior to the onset of these conditions.

Methods: A rat model of thioacetamide (TAA)-induced cholangiofibrosis was utilized to assess the impact of metformin on the induction process of cholangiocarcinoma (CCA). Liver tissues were harvested and analyzed histologically using light microscopy, complemented by a deep-learning convolutional neural network for enhanced evaluation. Additionally, RNA sequencing (RNA-seq) was performed to investigate the genetic alterations associated with metformin treatment in this TAA-induced cholangiofibrosis model.

Results: In the rat model, the TAA control group exhibited an increased incidence and average count of cholangiofibrosis cases in the liver, with rates of 100 % and an average of 12.0, compared to the metformin-treated group, which showed an incidence of 70 % and an average of 3.3. Notably, the progression from normal cholangioles to cholangiofibrosis was associated with the upregulation of several proteins critical for metabolic processes and the tumor microenvironment. These alterations were significantly mitigated by metformin treatment.

Abbreviations: Cholangiocarcinoma, CCA; Thioacetamide, TAA; Convolutional neural network, CNN; Whole slide image, WSI; Immunohistochemistry, IHC; Epithelial-mesenchymal transition, EMT.

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Conclusions: Long-term metformin use may offer protective benefits against cholangiofibrosis, partially by regulating metabolic processes and improving the tumor microenvironment.

1. Introduction

Worldwide, the incidence of cholangiocarcinoma (CCA) is fluid and complex [1–3]. The overall prognosis for most cases of CCA is dismal, and the median survival is 11.3 months for extrahepatic cholangiocarcinoma and 6.2 months for intrahepatic cholangiocarcinoma [4,5]. Several risk factors contribute to CCA, including primary sclerosing cholangitis, biliary stone disease, liver flukes, hepatitis C, and unhealthy lifestyles (smoking and alcohol) [6–8]. Therefore, chemoprevention of the damage caused by these risk factors has great potential in decreasing the incidence and mortality of CCA. Evidence from retrospective clinical studies suggests that the use of metformin is linked to a decreased risk of cancer development [9,10]. Metformin exerts its anti-inflammatory effects and modulates macrophage polarization through the activation of AMPK inhibits nuclear factor-κB activation, reduces the expression of inflammatory genes, and ultimately mitigates inflammatory injury [11,12]. Prevention with metformin in cohorts associated with a high risk for CCA may offer additional advantages concerning the risk-versus-benefit profile. However, the safety and duration of metformin as a chemopreventive agent are debated because prolonged use of metformin may lead to a deficiency in vitamin B12 (megaloblastic anemia) if left unchecked, which could lead to irreversible nerve damage [13,14]. This study was conducted to evaluate both the efficacy and safety of metformin in the prevention of CCA development.

To assess the impact of metformin on cancer development, we enrolled a rat model of cholangiofibrosis preceding the development of CCA, which was orally administered thioacetamide (TAA). This results in a chemically induced natural transition from normal cholangioles to biliary dysplasia and ultimately to cholangiofibrosis, a risk factor for CCA [15,16]. This model is useful for designing strategies for preclinical chemoprevention and therapeutic trials. Here, we utilized the TAA-induced cholangiofibrosis model to investigate whether metformin could prevent or diminish the incidence of cholangiofibrosis in vivo. Moreover, the potential mechanisms underlying metformin's inhibition of tumor cell growth were further investigated through cDNA microarray and RNA-seq analysis.

2. Materials & methods

2.1. Animal experimental protocol

10-week-old male Sprague-Dawley (SD) rats were used. To reduce potential variability associated with hormonal cycles, only male SD rats were selected for the metformin experiments aimed at preventing cholangiofibrosis. These animals were categorized into two groups: a control group receiving TAA alone (n = 16) and an experimental group receiving TAA in combination with metformin (n = 16). All rats were given 300 mg/L TAA (CAS: 27366-72-9, Hangyubiotech, Changzhou, China) mixed in their drinking water daily until they were sacrificed. Additionally, the experimental group received 250 mg/kg of metformin via intragastric administration every day, as informed by previous studies [17,18], whereas the control group was administered an equal volume. of regular water via intragastric administration for 20 weeks. Eight weeks after the initiation of TAA alone or TAA combined with metformin, two animals were harvested each month to assess the tumor-promoting effects of TAA and the safeguarding effects of metformin. After a total of 20 weeks, all rats were sacrificed for further evaluation. All rats were maintained under standard housing conditions.

2.2. Liver harvesting procedures and histopathological evaluation

At the designated time points, the animals were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally). A midline laparotomy was carried out to thoroughly inspect and assess all liver lobes for morphological changes and the incidence of cholangiofibrosis. Blood samples were obtained for subsequent analyses, including liver function tests and vitamin B12 deficiency assessments. The liver was then rinsed with normal saline and cut into sections measuring 3–5 mm at 5 mm intervals. Half of the liver tissue was stored at −80 °C for future studies, while the remaining portion was fixed in 4 % paraformaldehyde for histopathological examination.

2.3. Area ratio calculation

All slides of liver samples were digitized by Ningbo Jiangfeng biological information technology Co., Ltd. The tissue regions were extracted using the Otsu thresholding method, followed by morphological operations to refine the results, allowing for the calculation of the tissue areas (Atissue). To enhance lesion detection, we established an automated framework utilizing convolutional neural networks (CNNs) designed to identify and localize fibrosis and cholangiofibrosis regions within the whole slide images (WSIs). The detailed procedures were described in our previous report [19]. Finally, a ratio of liver lesions for each slide was generated: ratio = $A_{\text{lesion}}/A_{\text{tissue}}$.

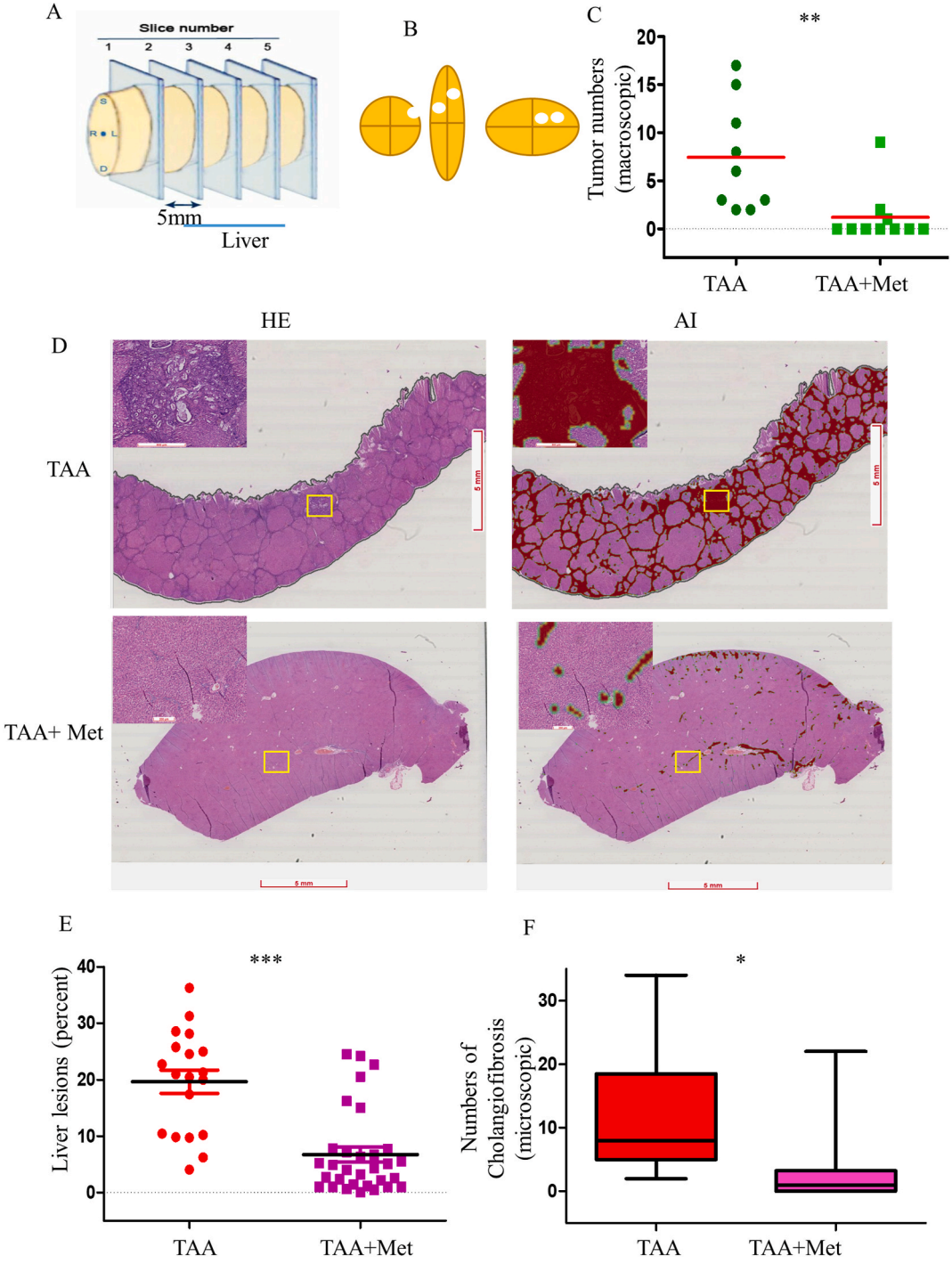

Fig. 1. The morbidity of cholangiolar proliferation and cholangiofibrosis in each group at 20 weeks. A, At 20 weeks, all rats were sacrificed and the livers were sliced into 5-mm pieces. B, Nodules on the surface of the liver and inside the liver were counted. C, Graphical representation of the numbers of visible nodules of each liver in the two groups. D, Representative images of the liver lesions from the two groups. Small figures are enlargement of the yellow box. HE, Hematoxylin-eosin staining; AI, Artificial intelligence. Small figures, 20 × ; large figure, 1 × . E, The ratio of liver lesions evaluated by AI in the two groups. F, The average numbers of microscopic cholangiofibrosis of the liver in the two groups. *P < 0.05, **P < 0.01, ***$P < 0.001$.

2.4. RNA-seq and data analysis

Sequencing and analysis were conducted on liver samples from four rats treated with TAA alone and five rats treated with both TAA and metformin. Total RNA was extracted using TRIzol reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen). RNA-seq libraries were prepared following the Illumina Standard Library Preparation Kit guidelines. The sequencing was performed on the Illumina HiScan™ 2500 platform at Shanghai Oe Biotech Co., Ltd. For quality control of the raw sequencing data, we utilized FastQC and NGS QC Toolkit v2.3.3. Clean reads were then aligned to the B73 reference genome (RefGen_v3) and the reference gene model dataset (FGS 5b) using TopHat/Bowtie2 (ccb.jhu.edu/software/tophat/). Gene expression levels were normalized as fragments per kilobase of exon model per million mapped reads (RPKM). A gene was deemed expressed if its PFKM value exceeded zero across all biological replicates. Differentially expressed genes were identified using a false discovery rate (FDR) threshold of <0.05, as determined by the DESeq software package (Bioconductor.org/) [19].

2.5. Immunohistochemistry (IHC) and assessment of liver specimens

Paraffin-embedded sections of TAA-induced tumors, cut to a thickness of four microns, were prepared for immunohistochemical analysis. Antibodies targeting S100A6 (H-55) and S100A10 (4E7E10) were sourced from Santa Cruz Biotechnology. Visualization of antibody binding was achieved using a streptavidin-biotin kit (#KIT-9720; Maixin-Bio). Two evaluators, G. Yu (a researcher) and Y. Chen (a pathologist), independently assessed all samples. The final outcomes were aggregated according to a semiquantitative scoring system, as outlined in previous studies [18,20].

2.6. Western blotting

Whole-cell lysates were obtained from the livers of rats in both the TAA and TAA + metformin groups. The lysates were subjected to SDS/PAGE for resolution and subsequently transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) via electrophoresis. Following the transfer, the membranes were incubated with the specified antibodies, and the resulting signals were detected using an enhanced chemiluminescence (ECL) kit from Santa Cruz (CA, USA).

2.7. Statistics

All statistical analyses were conducted using SPSS software (Chicago, IL) and GraphPad Prism (version 5.0). Categorical data were evaluated with χ^2 tests. To compare protein expression levels between normal and tumor tissues, we employed a two-tailed Student's t-test [20]. A P value below 0.05 was considered to indicate statistical significance.

3. Results

3.1. Systemic effects of TAA and TAA + metformin

The experimental outline for the evaluation of metformin-induced inhibition of TAA-induced cholangiofibrosis is shown in Fig. S1A. Throughout the 20-week study period, there were no occurrences of mortality related to TAA or metformin. Additionally, no significant differences were noted in body weight, liver condition, liver volume, or liver weight between the TAA group and the TAA + metformin group (Figs. S1B–D). Biochemistry test results, including those for AST, ALT, and vitamin B12, were similar for both groups (Fig. S1E).

3.2. The rate of nodule formation in the TAA and TAA + metformin groups

We began by carefully inspecting the white nodules on the liver's surface before proceeding to slice the liver into sections (Fig. 1A). The slices were subsequently evaluated for the number of white nodules present (Fig. 1B). Finally, we recorded the total number of nodules on both the liver surface and the slices. In the TAA group, the average number of macroscopic nodules was 7.4 ± 5.7, which was notably greater than the 1.2 ± 2.8 observed in the TAA + metformin group. (Fig. 1C).

Next, we conducted light microscopy and CNNs to detect and evaluate the liver sections. Generally, the liver size decreased, and the liver surface became rougher in the TAA group, whereas the liver surface in the TAA + metformin group appeared remarkably smooth (Fig. 1D). There were two obvious characteristics in the liver sections: cholangiolar proliferation and cholangiofibrosis, characterized by CK20-positive staining (Fig. S2A). We developed an AI algorithm to evaluate the ratio of cholangiolar proliferation as $A_{\text{lesion}}/A_{\text{tissue}}$ using deep-learning CNNs. This AI algorithm precisely located all cholangiolar proliferation (Fig. 1D) and provided an exact ratio (Fig. 1E). The ratio of cholangiolar proliferation in the TAA group was 19.6 ± 9.01, significantly greater than that in the TAA + metformin group (6.7 ± 7.4; Fig. 1D and E) ($P < 0.001$). Due to limited time, we didn't develop our AI algorithm to recognize cholangiofibrosis as a unique characteristic. Instead, we calculated the number of cholangiofibrosis visually. At 20 weeks, all rats in the TAA group exhibited cholangiofibrosis, and with 12 ± 10.1 microscopic cholangiofibrosis observed per rat. Strikingly, 70 % (7/10) of rats in the TAA + metformin group exhibited a rate of microscopic cholangiofibrosis of 3.3 ± 6.7 per rat, whereas the other 30 % did not display any development of cholangiofibrosis ($P < 0.05$) (Fig. 1F).

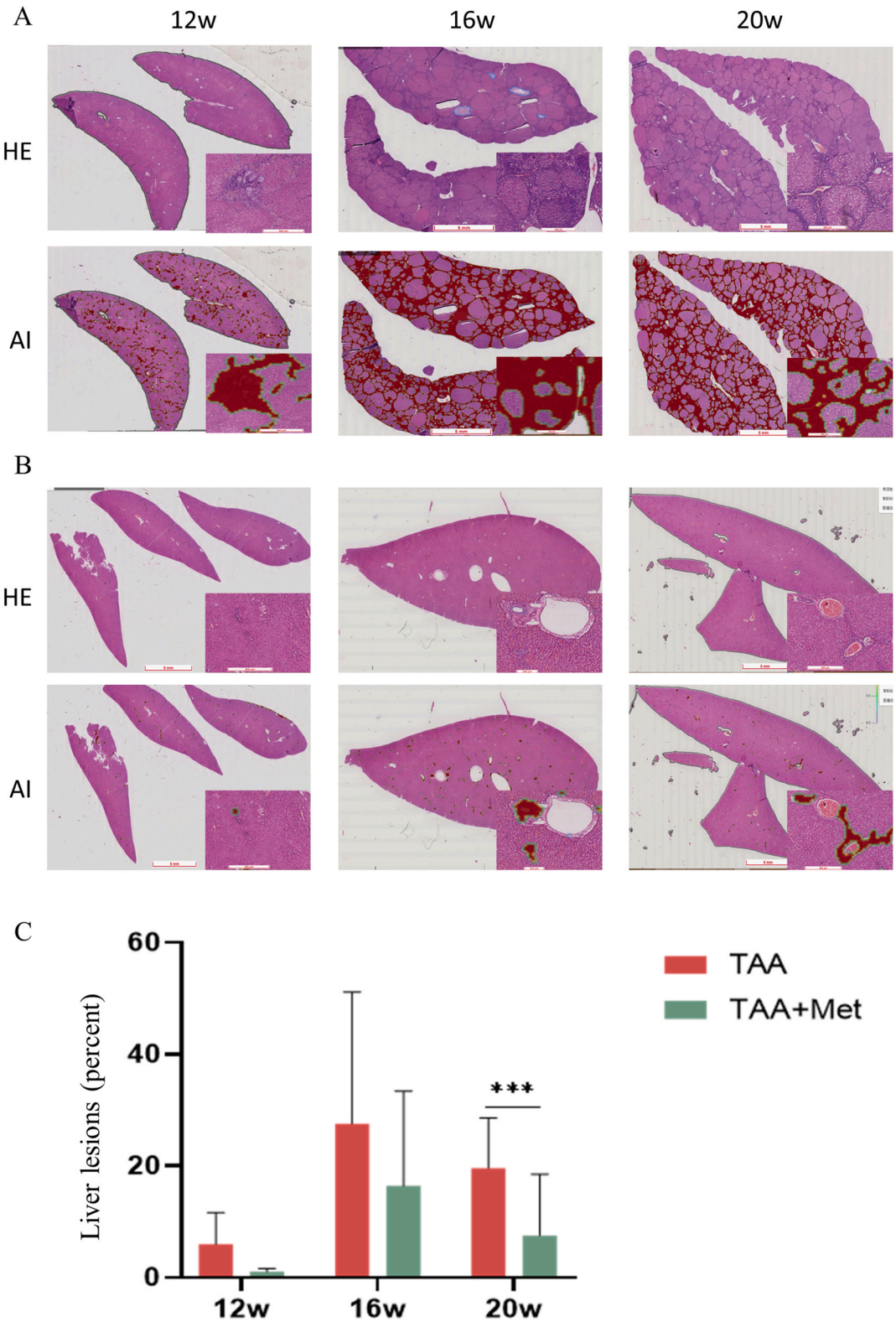

Fig. 2. The microscopic profiles of the liver lesions during TAA induction and/or metformin administration. A and B, Representative images of microscopic (upper) and AI-recognized (lower) cholangiolar proliferation of the liver in the TAA alone induction group (A) and in the TAA + metformin group (B). C, The average numbers of AI-recognized liver lesions from the TAA alone and the TAA + metformin group at the indicated time. ***$P < 0.001$.

3.3. Metformin inhibits TAA-induced cholangiolar proliferation

Next, we analyzed microscopic lesions of the liver during the 20-week study period. We mainly focused on the cholangiolar proliferation recognized through the AI algorithm. The proportion of microscopic cholangiolar proliferation in the TAA and TAA + metformin groups gradually increased with the administration of TAA revealed by both histological examination and AI (Fig. 2A and B). The baseline ratio of lesions in the TAA + metformin group (3.88 ± 2.78 at 12 weeks, 3.33 ± 1.91 at 16 weeks, 6.7 ± 7.4 at 20 weeks) was remarkably lower than that of the TAA group (5.98 ± 5.62 at 12 weeks, 27.57 ± 23.57 at 16 weeks, 19.6 ± 9.01 at 20 weeks) (Fig. 2C). These data further confirm that metformin retards the development of liver lesions induced by TAA.

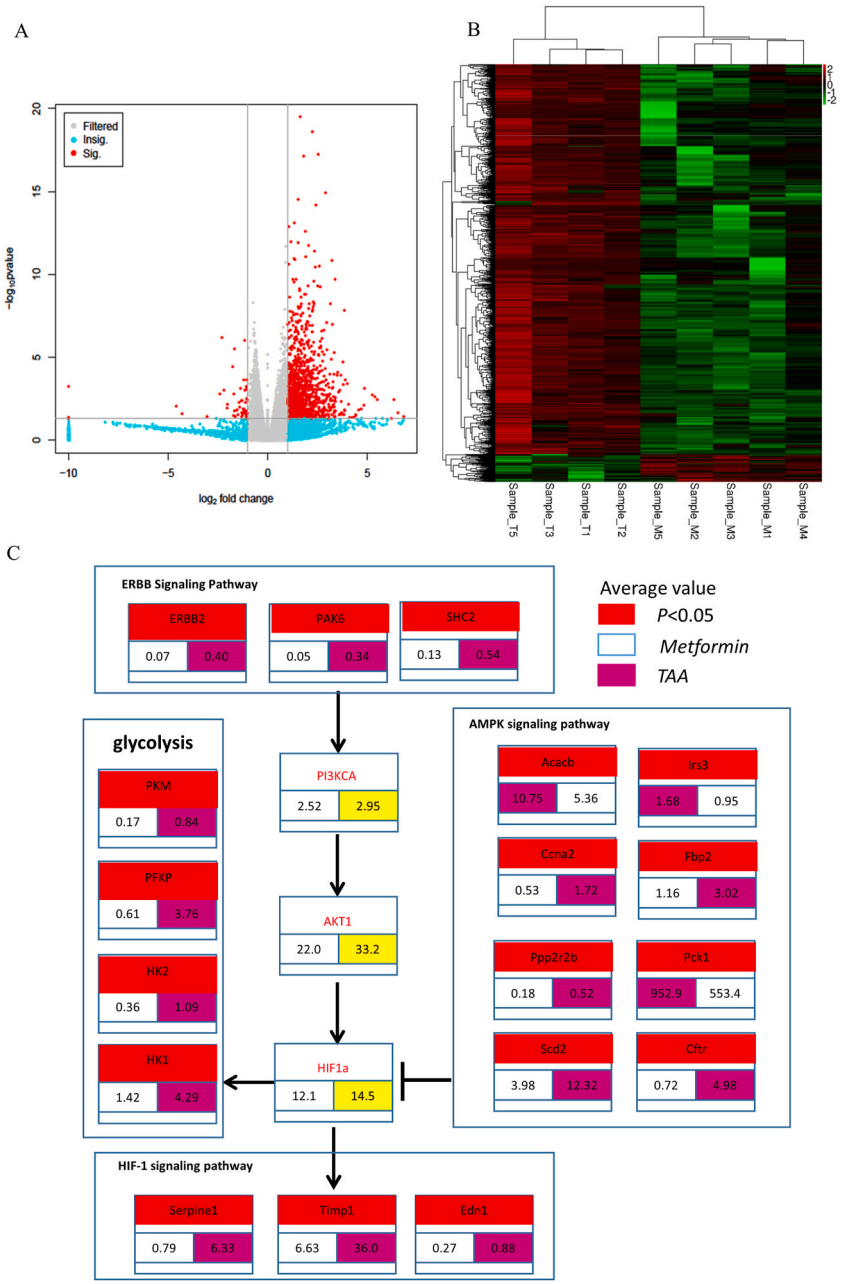

Fig. 3. RNA-seq analysis reveals differential gene expression between the livers from rats in the TAA group and the TAA + metformin group. A, Four livers from the TAA group and five livers from the TAA + metformin group were subjected to RNA-seq analysis and distributions of all genes are presented in the volcano map. B, Heatmap of significantly altered genes between the TAA group and the TAA + metformin group. C, Several proteins from multiple signaling pathways were significantly decreased upon metformin treatment compared that those without metformin treatment.

3.4. Metformin inhibits TAA-induced activation of multiple signaling pathways

Next, we explored the possible mechanisms underlying the metformin-dependent decrease in TAA-induced cholangiofibrosis using RNA-seq analysis. A number of genes were altered after long-term use of metformin compared with TAA induction alone (Fig. 3A and B). Metformin significantly influenced several signaling pathways, particularly those involving genes related to glycolysis, focal adhesion, and cancer progression (Fig. S2B). Notably, the ERBB2 signaling pathway was inhibited by metformin treatment. Furthermore, metformin treatment in TAA-induced cholangiofibrosis led to a 3- to 6-fold downregulation of key enzymes in glycolysis, such as hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PKM). Proteins in the HIF-1 signaling pathway, including Serpine1, Timp1, and Edn1, were also decreased by metformin treatment. Considering that the AMPK signaling pathway is the main target of metformin, we also examined the expression profiles of genes within this pathway. Three members, including Acacb, Irs3, and Pck1, showed increased expression upon metformin treatment, whereas another five members showed decreased expression upon metformin treatment (Fig. 3C).

Histopathology revealed that TAA-induced cholangiofibrosis were surrounded by intense stromal desmoplasia, along with inflammatory cell invasion [15]. Considering that S100 proteins have been shown to have a strong association with the tumor microenvironment (TME) [21], we investigated whether metformin treatment could influence their expression. Notably, several S100 proteins (S100A1, -4, -6, -8, -9, -10, and −11) were found to be highly expressed in the TAA group. Following metformin treatment, their expression levels were markedly decreased, showing a downregulation of 3- to 8-fold (Fig. 4A). Further IHC revealed that the expression levels of S100A6 and S100A10 was lower in specimens from the TAA + metformin group (66.7 ± 21.6 for S100A6 and 49.2 ± 15.6 for S100A10) than in the TAA group (141.7 ± 49.2 for S100A6 and 126.7 ± 41.8 for S100A10) ($P < 0.01$) (Fig. 4B). Moreover, metformin could inhibit the expression of S100A6 and S100A10 as revealed by western blotting (Fig. S3).

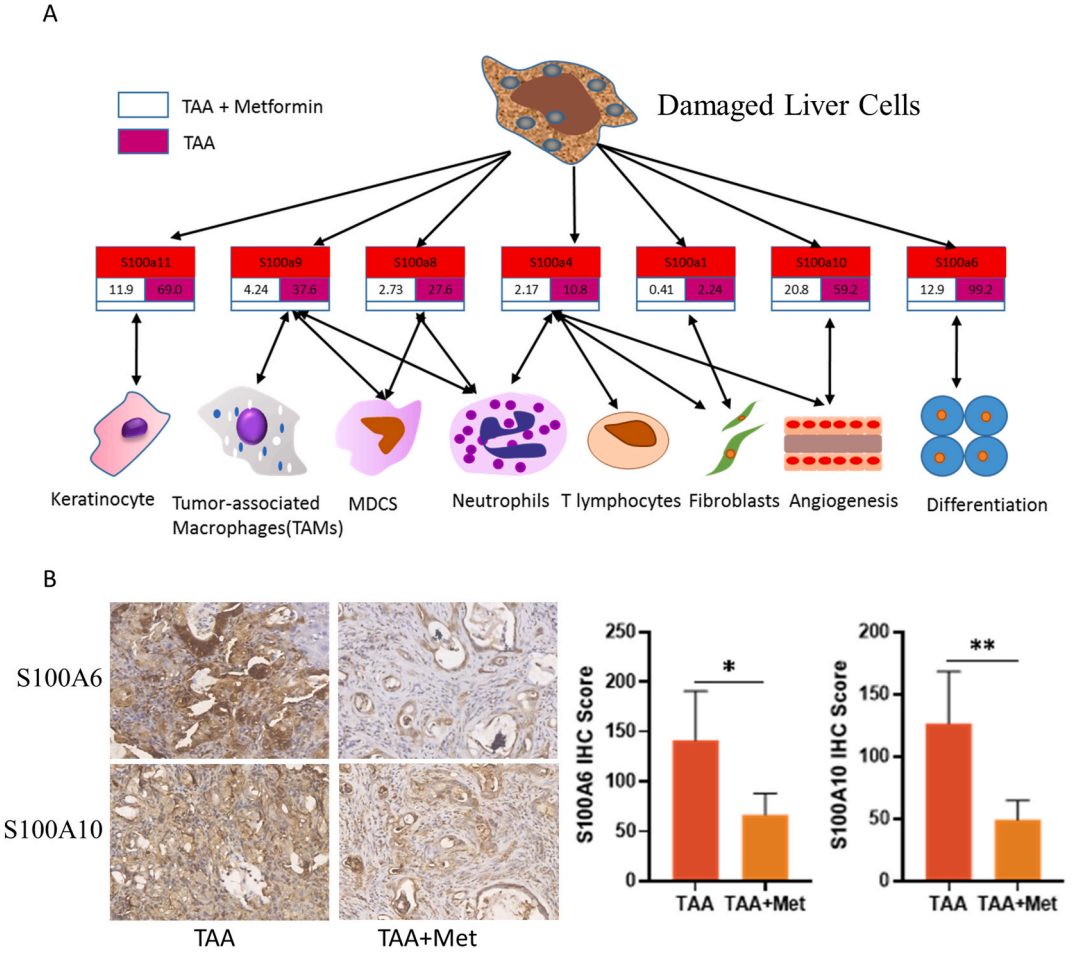

Fig. 4. Metformin improves liver microenvironment by decreasing the expression levels of several proteins in the S100 family. (A) A number of S100 proteins was significantly decreased upon metformin treatment compared that those without metformin treatment. (B) Low expression of S100A6 and S100A10 in liver lesions and tumors from TAA + Metformin group compared that from TAA group by IHC.

3.5. Mutation profiles between TAA group and TAA + metformin group

Considering the decreased incidence of cholangiofibrosis in rats and improved liver microenvironment induced by metformin, we investigated whether metformin treatment resulted in a low frequency of genetic aberrations. Unfortunately, neither the overall types of mutations nor missense mutations varied significantly between the two groups (Fig. S4). These data suggest that, once the mutations occur, they cannot be reversed, even if the tumor was controlled.

4. Discussion

The association of metformin with antineoplastic activity and its ability to reduce the burden of various cancers positions it as a promising therapeutic strategy for cancer treatment [22–24]. Metformin intake has been proved to improve clinical outcome in advanced cholangiocarcinoma patients after starting chemotherapy [25] and extend the survival of patients with advanced extrahepatic cholangiocarcinoma who have received drainage treatment [26]. However, metformin administration as an adjuvant therapy is debated in advanced solid cancers [27]. In particular, metformin treatment alone is unable to effectively inhibit the growth of various human solid cancer xenografts [17,28]. These results suggest that after malignancies become established, metformin might not offer any therapeutic potential [29]. However, increasing evidence suggests that metformin is supposed to be more effective for the chemoprevention of human solid cancers [29,30]. A phase 3 trial demonstrated the preventive effect of low-dose metformin (250 mg daily) in decreasing both the prevalence and the number of metachronous adenomas and polyps following polypectomy [31]. Given the relationship between metformin use and a decreased risk of ICC [32], we believe that metformin use prior to tumor establishment could decrease the incidence of TAA-induced cholangiofibrosis. Not surprisingly, long-term use of metformin indeed reduces the prevalence and number of cholangiofibrosis induced by TAA.

Metformin effectively inhibits cell growth by inducing cell cycle arrest and skewing macrophage polarization [33,34]. Moreover, not only are genes involved in cell cycle progression influenced by metformin, but genes associated with metabolic processes, response to stress, and protein transport or localization are also targeted by metformin [33]. Therefore, it is essential to explore the chemopreventive role of metformin in CCA. TAA-induced cholangiofibrosis is an ideal model for our study because this model produces a 100 % rate of cholangiofibrosis, a risk factor for CCA, by the 22 nd week of treatment. TAA is a potent hepatotoxin that interferes with ribosomal activity, thus hindering protein synthesis [35] and stimulating DNA synthesis [36].

Metformin use as a chemopreventive agent has been widely explored. Here, we introduced an AI algorithm to assess all H&E slides [37], avoiding the subjective judgment from the pathologists. Surprisingly, prolonged administration of metformin led to a 30 % reduction in TAA-induced cholangiofibrosis in all rats and decreased the incidence of tumor-like nodules in each rat treated with metformin. In addition to visible nodules, another major characteristic of our carcinogenesis model is cholangiolar proliferation [15]. Long-term use of metformin could improve the quality of the liver tissue by reducing the area of cholangiolar proliferation. Thus, the 'soil' of the liver destroyed by TAA will be restored by metformin. Importantly, we observed no side effects, such as gastrointestinal disturbances or vitamin B12 deficiency, associated with the long-term use of metformin when vitamin B12 was supplemented through dietary sources.

The mechanisms through which metformin exerts its effects have been extensively studied. Notably, metformin has the potential to

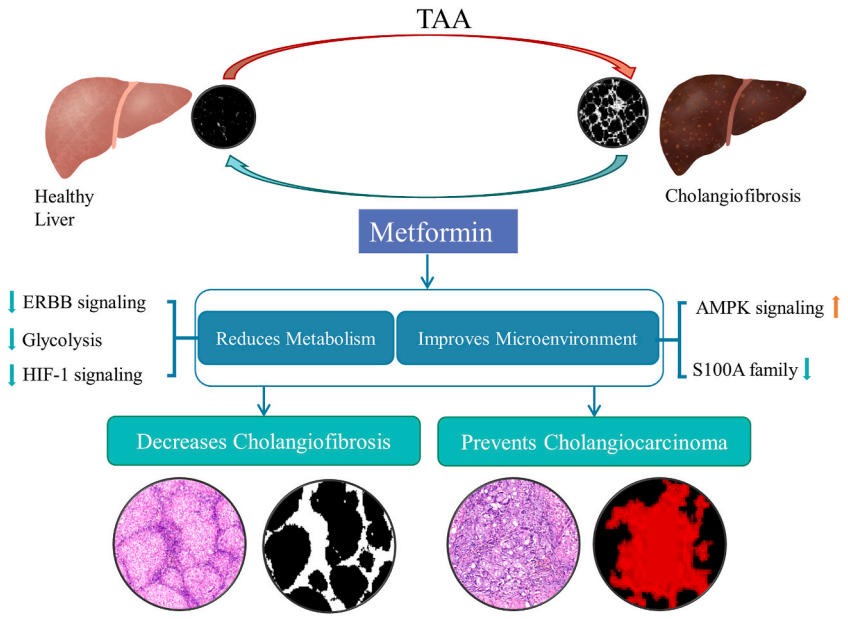

Fig. 5. A diagrammatic figure summarizing the main findings and conclusions for the current proposal.

reverse mesenchymal and epithelial-mesenchymal transition (EMT) characteristics in intrahepatic CCA by activating AMPK-FOXO3-related pathways [24]. Here, we expanded its potential mechanism against TAA-induced liver diseases (Fig. 5). Given that metformin treatment did not lower the frequency of genetic aberrations, its chemopreventive effects may be attributed to the functional alterations resulting from genetic mutations. ERBB2 is overexpressed in invasive CCAs, and upon metformin treatment, ERBB2 was down-regulated in our cDNA and RNA-seq datasets. In addition to ERBB2, two other members of the ERBB signaling pathway, PAK6 and SHC2, were also decreased upon metformin treatment. Similarly, members of the HIF signaling pathway were also down-regulated upon metformin treatment. Tumor cells can exhibit glycolytic rates that are over 200 times greater than those of the normal tissues from which they originate. Interestingly, treatment with metformin resulted in decreased expression of key rate-limiting enzymes in glycolysis, such as HK, PFK, and PKM. This suggests a novel mechanism by which metformin may prevent cancer by reducing glycolysis and effectively starving cancer cells. As an activator of the AMPK signaling pathway, metformin administration influenced the expression of over eight genes within this pathway in rats with TAA-induced cholangiofibrosis. Additionally, the S100 protein family plays diverse roles in both intracellular and extracellular environments, particularly in relation to the tumor microenvironment [21, 38]. It is well recognized that the S100 family facilitates communication between cancer cells and stromal cells. Our finding that long-term metformin use significantly reduces the expression of various S100 proteins helps explain the relatively improved liver condition observed in rats from the TAA + metformin group compared to those receiving TAA alone. However, the evidence suggesting metformin's role in inhibiting tumorigenesis remains indirect. Therefore, additional research is required to elucidate the direct impact of metformin on these signaling pathways.

However, this study has several limitations. First, the dosages of metformin used in the animal protocol are relatively high compared to human clinical exposure. Further research should systematically evaluate a range of dosages to identify the minimum effective dose and better correlate these findings with human clinical levels. Second, the role of sex hormones in mediating the response to metformin treatment has not been investigated. To address this, we plan to conduct follow-up studies that include both male and female mice to examine any sex-specific effects. Additionally, more relevant animal models that closely mimic the progression to cholangiocarcinoma (CCA) [39], including genetically engineered mouse models, are necessary to confirm metformin's preventive and therapeutic effects in both early and advanced stages of the disease. Moreover, the precise mechanism by which metformin inhibits tumor initiation remains unclear. An in-depth investigation of its mechanisms and a more rigorous cohort study will be essential for future research.

In summary, our data demonstrate for the first time that the use of metformin is effective in inhibiting or delaying TAA-induced biliary disease in rats by targeting multiple signaling pathways essential for tumor formation. This evidence provides a valuable scientific basis for the potential contribution of metformin to the chemoprevention of tumors, especially for those with high-risk factors.

Ethics statement

All animal protocols for this study received approval from the Experimental Animal Ethics Committee at Longhua Hospital, which is affiliated with Shanghai University of Traditional Chinese Medicine, China (Approval number: PZSHUTCM210514001).

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Data availability statement

The RNA-seq datasets featured in this study are available in online repositories. The repository names and corresponding accession numbers can be accessed at: https://www.ncbi.nlm.nih.gov/bioproject/795823.

CRediT authorship contribution statement

Chaofu Li: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Yating Deng:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yating Pan:** Methodology, Investigation, Formal analysis, Data curation. **Xinyi Liao:** Methodology, Investigation. **Huadong Xie:** Investigation, Data curation. **Xiaoli Xue:** Methodology, Data curation. **Shaoqing Yu:** Supervision, Software, Resources, Methodology, Conceptualization. **Wenlong Yu:** Software, Project administration, Methodology, Conceptualization. **Guanzhen Yu:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37347.

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