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TGFβ-induced circLTBP2 predicts a poor prognosis in intrahepatic cholangiocarcinoma and mediates gemcitabine resistance by sponging miR-338-3p

Authors

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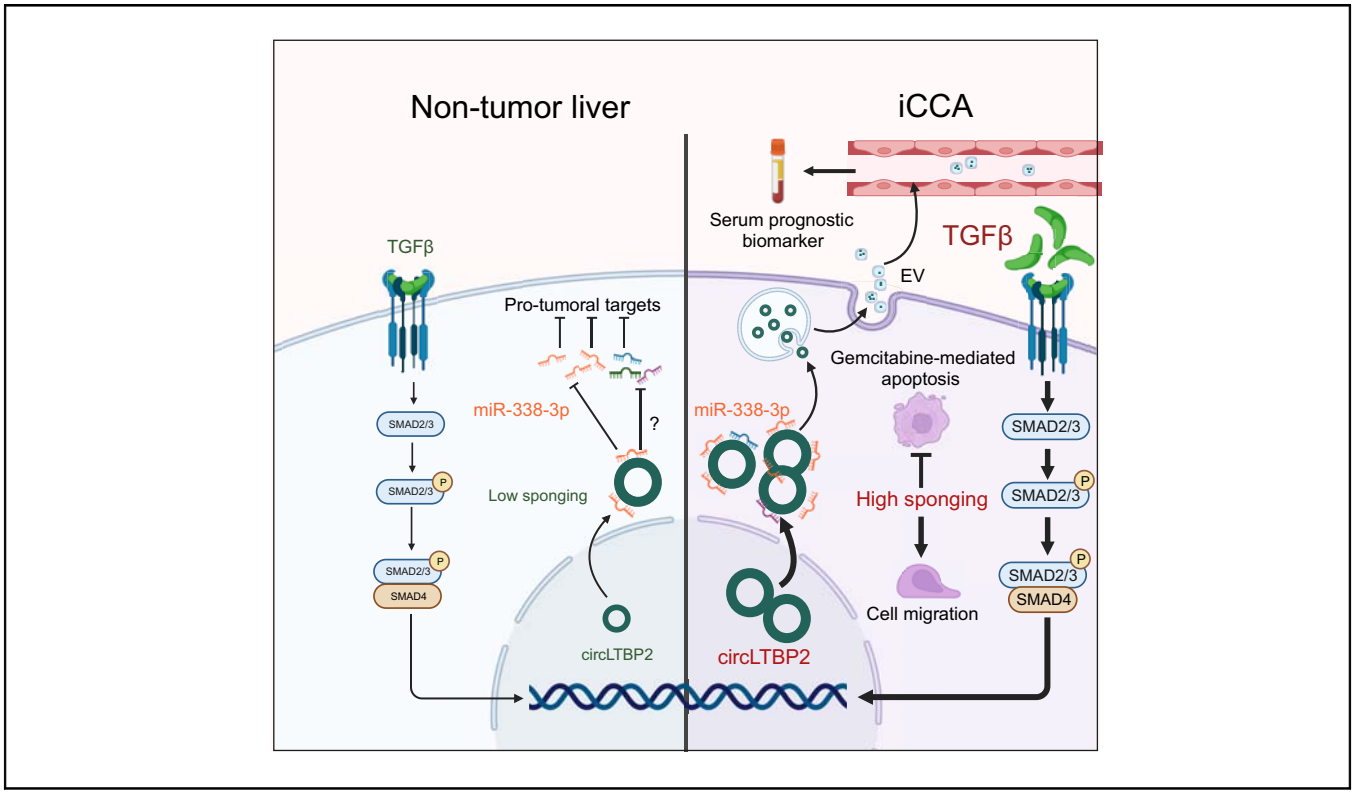
Graphical abstract



Highlights

- CircLTBP2 is induced by TGFβ in CCA cells, and its expression in CCA tumour tissues predicts a poor prognosis.
- CircLTBP2 could serve as a diagnostic biomarker differentiating iCCA from HCC.
- CircLTBP2 promotes cell proliferation, migration, and resistance to gemcitabine-mediated apoptosis.
- CircLTBP2 exerts pro-tumoural actions by sponging miR-338-3p.
- CircLTBP2 is detected in liquid biopsies and predicts a poor prognosis.

Impact and implications

Intrahepatic cholangiocarcinoma (iCCA) is an aggressive cancer with limited therapeutic options. Opening the field to new concepts is urgently needed to improve the survival of patients. Here, we evaluated the role and the clinical relevance of circular RNA. We report that TGFβ-induced circLTBP2 contributes to CCA carcinogenesis and may constitute a clinically relevant prognostic biomarker detected in liquid biopsies.

https://doi.org/10.1016/j.jhepr.2023.100900

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TGFβ-induced circLTBP2 predicts a poor prognosis in intrahepatic cholangiocarcinoma and mediates gemcitabine resistance by sponging miR-338-3p

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JHEP Reports **2023.** https://doi.org/10.1016/j.jhepr.2023.100900

Background & Aims: Intrahepatic cholangiocarcinoma (iCCA) is a deadly cancer worldwide with an increasing incidence and limited therapeutic options. Therefore, there is an urgent need to open the field to new concepts for identifying clinically relevant therapeutic targets and biomarkers. Here, we explored the role and the clinical relevance of circular RNA (circRNA) circLTBP2 in iCCA.

Methods: Transforming growth factor β (TGFβ)-regulated circRNAs were identified by dedicated microarrays in human HuCC-T1 iCCA cell line, and their clinical relevance was evaluated in independent cohorts of patients. Gain and loss of function of circLTBP2 combined with functional tests was performed *in vitro* and *in vivo* in mice. RNA pulldown, microRNA sequencing, and RNA immunoprecipitation were performed to explore the sponging activity of circLTBP2.

Results: CircLTBP2 (has_circ_0032603) was identified as a novel TGFβ-induced circRNA in several cholangiocarcinoma cell lines. CircLTBP2 promotes tumour cell proliferation, migration, and resistance to gemcitabine-induced apoptosis *in vitro* and tumour growth *in vivo*. Mechanistically, circLTBP2 acts as a competitive RNA regulating notably the activity of the tumour suppressor microRNA miR-338-3p, leading to the overexpression of its pro-metastatic targets. The restoration of miR-338-3p levels in iCCA cells reversed the pro-tumourigenic effects driven by circLTBP2, including the resistance to gemcitabine-induced apoptosis. In addition, circLTBP2 expression predicted a reduced survival, as detected in not only tumour tissues but also serum extracellular vesicles isolated from patients with iCCA.

Conclusions: CircLTBP2 is a novel effector of the pro-tumourigenic arm of TGFβ and a clinically relevant biomarker easily detected from liquid biopsies in iCCA.

Impact and implications: Intrahepatic cholangiocarcinoma (iCCA) is an aggressive cancer with limited therapeutic options. Opening the field to new concepts is urgently needed to improve the survival of patients. Here, we evaluated the role and the clinical relevance of circular RNA. We report that TGFβ-induced circLTBP2 contributes to CCA carcinogenesis and may constitute a clinically relevant prognostic biomarker detected in liquid biopsies.

Introduction

Cholangiocarcinoma (CCA) includes a heterogeneous group of tumours that can occur anywhere in the biliary system. CCA is a rare cancer, but both its incidence and mortality increased worldwide over the past decade.[1,2] The silent presentation of CCA, combined with its aggressive nature and resistance to chemotherapy, contributes to the high mortality rate of CCA, which represents 2% of all cancer-related deaths worldwide each year. The current methods for diagnosing CCA are not accurate enough, and the high heterogeneity of CCA severely limits the effectiveness of therapies.[3] Therefore, there is an urgent need of opening the field to new concepts to identify relevant therapeutic targets and biomarkers able to improve the survival of patients with CCA.[4,5]

Recently, circular RNAs (circRNAs) have emerged as new regulators of cancer-related processes, such as cell proliferation, migration, and drug resistance, acting notably as sponges for microRNAs (miRNAs).[6] CircRNAs are abundant and evolutionarily conserved RNA molecules generated by a non-canonical back-splicing. They have been found to be aberrantly expressed in several types of cancer, making them potential biomarkers for early detection and prognosis. These recent findings highlight

Keywords: Cholangiocarcinoma; Circular RNA; Transforming growth factor beta; Biomarker; Extracellular vesicles.

Received 22 March 2023; received in revised form 15 August 2023; accepted 30 August 2023; available online 5 September 2023

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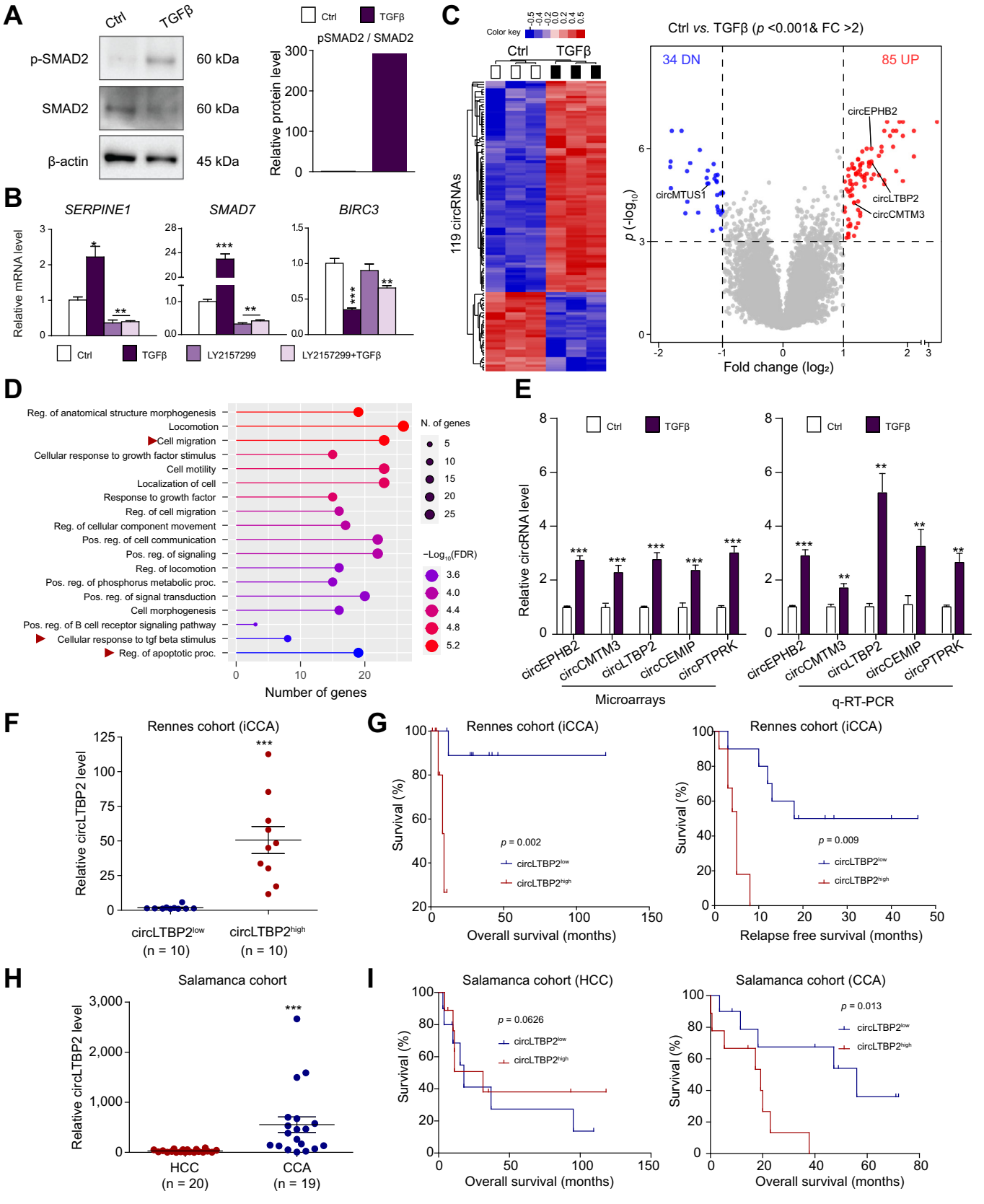


Fig. 1. CircLTBP2, a TGFβ-induced circRNA, predicts a poor prognosis in patients with iCCA. (A) Western blot analysis of p-SMAD2 and SMAD2 expression in HuCC-T1 iCCA cell line in response to TGFβ (1 ng/ml TGFβ, 16 h). (B) Expression of three well-known TGFβ target genes in HuCC-T1: *SERPINE1*, *SMAD7*, and *BIRC3*. Cells were treated with TGFβ (1 ng/ml) alone or in combination with LY2157299 (galunisertib) (10 μM) for 16 h. (C) Hierarchical clustering and volcano plot of circRNA isoforms differentially expressed by TGFβ (1 ng/ml, 16 h) in HuCC-T1. A specific signature of 119 circRNAs, upregulated (UP, n = 85) or downregulated (DN,

the importance of circRNAs in cancer biology and their potential as targets for the development of new treatments.[7] However, there are currently very few studies in intrahepatic CCA (iCCA).[8,9] For example, circACTN4 and circGGNBP2 were recently reported as regulators of iCCA progression, modulating specific signalling pathways, including Hippo and Wnt/β-catenin.[10–12] The transforming growth factor beta (TGFβ) pathway also contributes to CCA carcinogenesis, but the role of circRNAs as effectors of TGFβ remains unexplored so far in CCA. Accordingly, this study aims at evaluating the role and the clinical relevance of TGFβ-regulated circRNAs in iCCA.

Materials and methods

Patients and tissue samples

Freshly frozen human CCA samples were obtained from the National Liver Network (BB-0033-00085). A validating cohort, which included 12 iCCA, five distal CCA, two perihilar CCA, and 20 hepatocellular carcinoma (HCC), was also used. Serum samples were obtained from patients with advanced metastatic iCCA (n = 62) from the CLCC Eugène Marquis (Rennes, France).

Cell lines and functional assays

HuCC-T1 (RCB-1960) and HuH-28 (RCB-1943) CCA cell lines were obtained from the RIKEN BioResource Center in Japan. SG231, Mz-ChA-1, TFK-1, and SK-ChA-1 were kindly provided by Dr. Laura Fouassier from the Centre de Recherche Saint-Antoine (Inserm U938, Paris, France). Culture conditions are described in the Supplementary Materials and Methods.

Full-length circLTBP2 (has_circ_0032603) was cloned into pLV-circ_GFP by Creative Biogene (17 Ramsey Road, Shirley, NY 11967, USA) to perform gain-of-function (GOF) experiments. Antisense LNA GapmeRs that target the circularisation junction of circLTBP2 (#339511, Qiagen) were used for loss-of-function (LOF) experiments. Cell proliferation, migration, and apoptosis assays were performed using an Incucyte S3 device (Sartorius, USA) as described in the Supplementary Materials and methods.

CircRNA microarray analysis

Total RNA, including circRNAs, was purified using an miRNeasy kit (Qiagen, #217004). Arraystar Human circRNA Microarrays v2.0 (Arraystar, Rockville, MD, USA) were used to screen for novel TGFβ-regulated circRNAs in human CCA cell lines.

q-RT-PCR, Western blot, and IP

Quantitative reverse-transcription PCR (q-RT-PCR) and Western blot were performed as previously described.[13] Primers and antibodies are listed in Table S1. MiRNA immunoprecipitation (IP) was carried out according to the manufacturer's instructions (miRNA Target IP Kit #25500, Active Motif, Inc.). Briefly, miR-338-3p mimics (miRCURY LNA miRNA Mimics, Qiagen, 5 nM) were transfected into HuCC-T1 cells for 24 h. The IP was performed using a pan-Ago antibody to identify miRNA/mRNA and miRNA/circRNA complexes.

CircRNA pulldown assay

A circLTBP2 pulldown assay was carried out by Creative Biogene. Briefly, a labelled circRNA expression vector was generated and cotransfected into HuCC-T1 cells with a capture protein expression vector. Afterwards, a pulldown assay was done by using a labelled antibody that binds to the induced capture protein, which allows for the pulldown of circLTBP2–miRNA complexes. Eluted miRNAs were identified by miRNA sequencing.

Tumour xenograft model

HuCC-T1 cells were modified to overexpress either an empty vector (pLV-circRNA-GFP) or circLTBP2 (pLV-circLTBP2-GFP) and were infected with GL261-Luc (CMV-Firefly luciferase lentivirus (Neo), PLV-10064-50, Cellomics Technology, USA). A total of 4,000,000 cells were implanted on the flanks of 8-week-old female NSG mice (Charles River, USA). All animal procedures followed the European Community Directive guidelines (Agreement B35- 639 238-40, Biosit, Rennes, France; DIR #7163) and were approved by the local ethics committee. Tumour growth was evaluated by measuring the size of the tumour using a caliper. Then, 90 days after implantation, mice were sacrificed, and the tumours, liver, and lung tissues were collected and analysed at the molecular level. The presence of metastases in the liver and lungs was evaluated by bioluminescence.

Statistical analyses

All statistical analyses were conducted using Prism 8 software (GraphPad Software, Boston, MA, USA), and the results were presented as mean values ± SD, or median values. Comparison between variables was made using either Student *t* test or the Mann-Whitney *U* test. For multiple sets of multivariate comparisons, two-way ANOVA was applied. Survival curves were analysed using the Kaplan-Meier method and log-rank test. A *p* value of <0.05 was considered statistically significant.

Results

HuCC-T1 is a TGFβ-responsive iCCA cell line

CCA is a rare cancer with limited characterised *in vitro* models.[14] Therefore, we first characterised three iCCA (HuCC-T1, HuH-28, and SG231) and three extracellular CCA (Mz-ChA-1, TFK-1, and SK-ChA-1) cell lines. Mz-ChA1 and TFK-1 displayed an epithelial phenotype (high expression of E-cadherin [CDH1] and no expression of vimentin [VIM]), whereas HuH-28 displayed a mesenchymal phenotype (high VIM/no CDH1). SK-ChA-1, SG231,

n = 34) by TGFβ was identified. Horizontal dashed line on the volcano plot: p <0.001. Vertical dashed lines: FC >2. (D) Gene ontology analysis based on circRNA microarray signature showed that dysregulated genes upon TGFβ treatment are involved in the regulation of cell migration and response to apoptosis. Values of p were calculated using the permutation test. (E) Five TGFβ-induced circRNAs randomly selected and their relative expression, as determined by microarray and q-RT-PCR. (F) CircLTBP2 (has_circ_0032603) relative expression in 20 freshly frozen iCCA tumours from a French national cohort (separated in two groups, namely, circLTBP2^{low} and circLTBP2^{high}, according to their circLTBP2 median expression). (G) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased OS and relapse-free survival for patients with iCCA with a high expression of circLTBP2. (H) CircLTBP2 relative expression in HCC and CCA tumours unravelled circLTBP2 specificity for CCA. (I) Kaplan-Meier plots and log-rank statistics analysis endorsed the association of circLTBP2 expression with OS in the independent cohort of CCA, whereas no correlation was found in HCC. Statistical analyses for (A–C) were performed using a Mann-Whitney *U* test. Data are presented as mean ± SD. *p <0.05, **p <0.01, ***p<0.001, n ≥3 technical and biological replicates. CCA, cholangiocarcinoma; circRNA, circular RNA; FC, fold change; FDR, false discovery rate; HCC, hepatocellular carcinoma; iCCA, intrahepatic CCA; LTBP2, latent TGFβ-binding protein 2; OS, overall survival; q-RT-PCR, quantitative reverse-transcription PCR; TGFβ, transforming growth factor β.

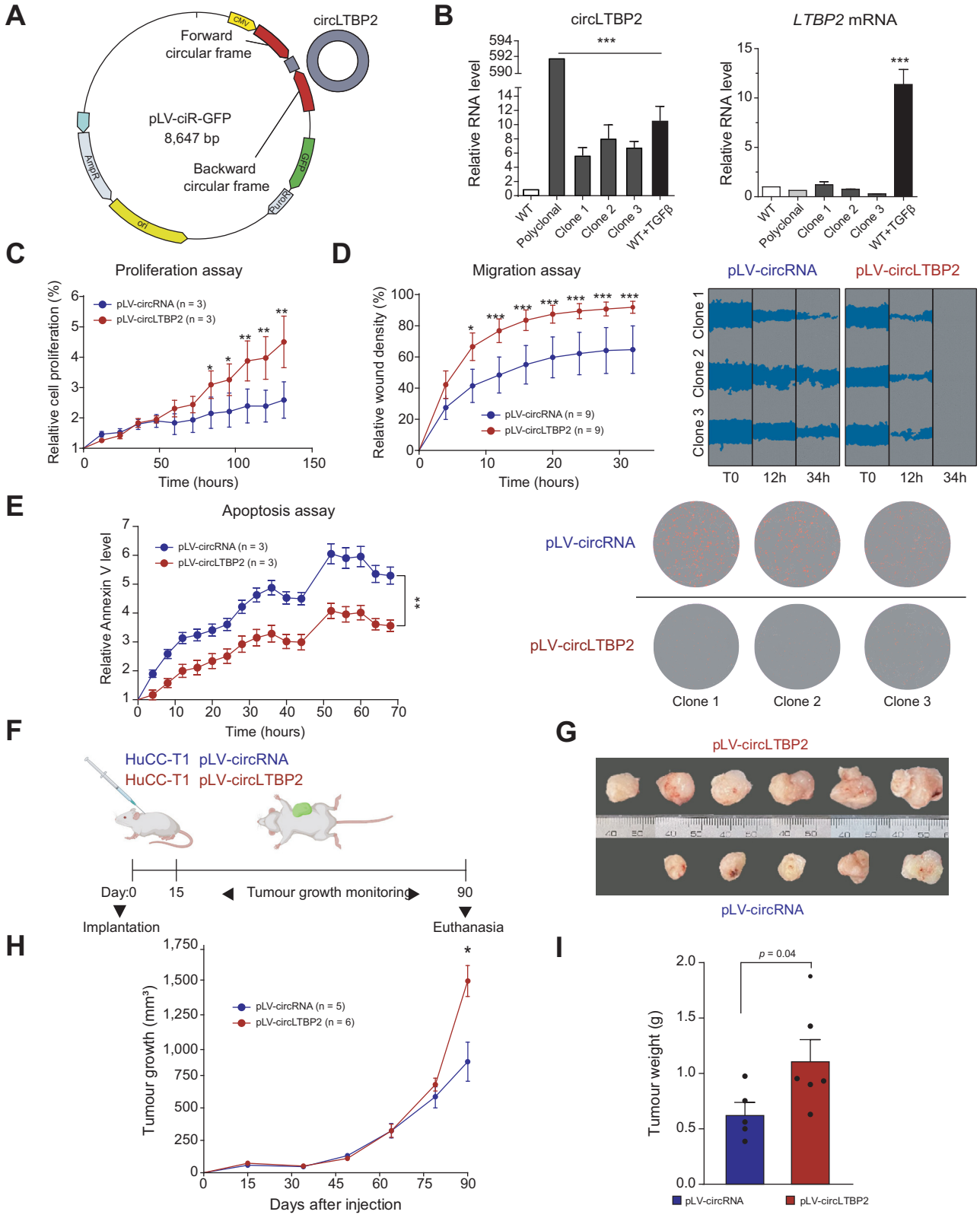


Fig. 2. Overexpression of circLTBP2 promotes iCCA cell proliferation, migration, and resistance to gemcitabine-induced apoptosis *in vitro* and tumour growth *in vivo*. (A) Schematic representation of circLTBP2 overexpression vector. (B) Relative expression of circLTBP2 and its cognate mRNA in HuCC-T1 clones infected with circLTBP2 overexpression vector. (C) Relative confluency of cultured HuCC-T1 cells was evaluated for 132 h (n = 3 per group). HuCC-T1 cells overexpressing circLTBP2 showed an increased proliferation. (D) Cell migration was evaluated by wound healing assay performed on HuCC-T1 cells overexpressing circLTBP2 compared with the empty vector control group (n = 9 per group). Relative wound density was assessed every 4 h as a percentage of the initial wound surface, represented in blue on the right panel. HuCC-T1 cells were cultured in low FBS concentration medium (1% FBS) and with mitomycin C

and HuCC-T1 exhibited an intermediate phenotype with expression of both CDH1 and VIM (Fig. S1A–C). Next, we evaluated their response to TGFβ by measuring the level of phosphorylated SMAD2 (Fig. 1A and Fig. S1D) and the expression of TGFβ target genes (*SERPINE1*, *SMAD7*, and *BIRC3*). HuCC-T1 and HuH-28 cell lines were fully responsive to TGFβ, with a significant SMAD2 phosphorylation, upregulation of *SERPINE1* and *SMAD7*, and downregulation of *BIRC3*. In addition, these effects were reversed in the presence of galunisertib (LY2157299), a selective inhibitor of TGFβ receptor type 1 (TGFBR1) (Fig. 1B and Fig. S1E). Subsequent experiments were performed in HuCC-T1 cells, which exhibited better culture conditions and transfection efficiency.

CircLTBP2 is a TGFβ-induced circRNA predictive of poor prognosis in iCCA

Gene expression profiling using Arraystar microarrays identified 119 circRNAs significantly (p <0.001 and fold change >2) deregulated by TGFβ (1 ng/ml, 16 h) in HuCC-T1, including 85 upregulated and 34 downregulated circRNAs (Fig. 1C and Table S2). Interestingly, more than 50% of circRNAs and their linear counterparts were coregulated in response to TGFβ (Fig. S2). Out of the 119 TGFβ-regulated circRNAs, 43 were predicted to act as miRNA sponges (Fig. S2). A gene ontology analysis revealed that these circRNAs were associated to biological processes regulated by TGFβ, such as cell migration and apoptosis (Fig. 1D). The expression of five randomly selected TGFβ-induced circRNAs was validated by q-RT-PCR (Fig. 1E). Next, we evaluated their clinical relevance in resected iCCA tumours (cohort 1, n = 20). Among the five candidates, circLTBP2 was associated with a poor prognosis as its expression was predictive of a significant reduced overall (OS) and relapse-free (RFS) survival (p <0.01) (Fig. 1F and G, and Fig. S3A). The association of circLTBP2 expression and OS was further validated in two additional cohorts of liver cancers, including HCC and CCA (Fig. S3B and C, and Fig. 1I). Interestingly, the expression of circLTBP2 was very low in HCC and not associated with OS (Fig. 1H and I). CircLTBP2 was detected in both iCCA and extrahepatic CCA tissues and induced by TGFβ in all CCA cell lines with the exception of SG231 (Fig. 1H and Fig. S4). CircLTBP2 is a large exonic circRNA (2,165 bases) composed of exons 2–16 of latent TGFβ-binding protein 2 (*LTBP2*) mRNA. Its circular structure was confirmed by Sanger sequencing (Fig. S5A). We developed sets of convergent and divergent primers to specifically detect the circLTBP2 isoform hsa_circ_0032603, its linear LTBP2 counterpart, and housekeeping circRNA controls (Supplementary Methods, and Figs. S5A–C and S6). In HuCC-T1 cells, circLTBP2 induction by TGFβ was the highest (Fig. S4) and abolished in the presence of galunisertib or SMAD3 inhibitor SIS3 (Fig. S5D and E).

CircLTBP2 exerts pro-oncogenic actions in iCCA

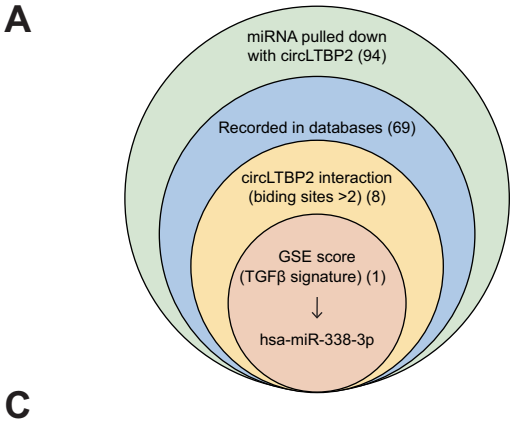
First, we performed GOF experiments by infecting HuCC-T1 cells with a vector that contained circLTBP2 (pLV-circLTBP2) (Fig. 2A). Overexpression was validated in several HuCC-T1 clones, and three of them demonstrating similar levels of circLTBP2 expression as seen after TGFβ treatment were selected for functional assays. Importantly, the expression of linear LTBP2 was not affected by circLTBP2 overexpression (Fig. 2B). TGFβ contributes to CCA progression by modulating cell proliferation, migration, and apoptosis.[15] Thus, we investigated the impact of circLTBP2 on these processes using an Incucyte S3 device. HuCC-T1 cells stably overexpressing circLTBP2 exhibited significantly higher proliferation and migration abilities than cells infected with an empty vector (Fig. 2C and D). In addition, circLTBP2 overexpression promoted resistance to gemcitabine-induced apoptosis (Fig. 2E). The pro-tumourigenic potential of circLTBP2 was further evaluated *in vivo* by injecting genetically engineered HuCC-T1 cells into NSG mice (Fig. 2F). Tumours developed in mice injected with cells overexpressing circLTBP2 were significantly bigger in size (856 ± 336 *vs*. 1,478 ± 292 mm^3, p <0.001) and weight (0.60 ± 0.21 *vs*. 1.12 ± 0.41 g, p <0.05) (Fig. 2G–I). CircLTBP2 overexpression in the resected tumours was confirmed by q-RT-PCR (Fig. S7A). Even though circLTBP2-overexpressing tumours exhibited a mesenchymal phenotype (VIM+ and CDH1-, as evaluated by immunohistochemistry), no difference was observed in the number or size of lung metastases (Fig. S7B).

Next, LOF experiments using LNA GapmeRs specifically targeting circLTBP2 circularisation junction were performed (Fig. S7C–G). Importantly, these GapmeRs were shown to specifically decrease the expression of circLTBP2 without impacting the expression of its linear counterpart (Fig. S7D). Validating our previous observation, circLTBP2 LOF in HuCC-T1 cells resulted in a decrease of cell proliferation and migration, and an improved sensitivity to gemcitabine (Fig. S7E–G). In addition, the data demonstrated that TGFβ slightly but significantly reduced cell proliferation and increased cell migration and resistance to gemcitabine-induced apoptosis. These effects were abolished in the presence of GapmeRs targeting circLTBP2 (Fig. S7E–G). Interestingly, a decreased cell proliferation upon TGFβ treatment and transfection of circLTBP2 GapmeRs was also highlighted in TFK1 cells, suggesting that circLTBP2 may counteract the cytostatic effects of TGFβ (Fig. S8).

CircLTBP2 acts as a sponge for miR-338-3p, a tumour suppressor miRNA

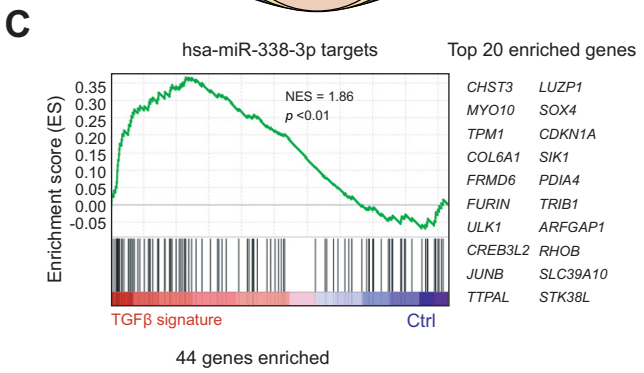
To gain insight into the molecular mechanisms driving the effects of circLTBP2, we performed a circLTBP2 pulldown assay followed by miRNA sequencing. Owing to the large size of circLTBP2 and the diverse functional impact of circLTBP2, we hypothesised that circLTBP2 may act as a sponge for tumour suppressor miRNAs. A decision-making pattern was established to select the most promising miRNA candidates (Fig. 3A). By circRNA pulldown, we identified 94 miRNAs possibly interacting with circLTBP2, and among them, 69 were recorded in databases. The selection of miRNAs also relied on a strong dependency to circLTBP2, as determined by the number of predicted binding

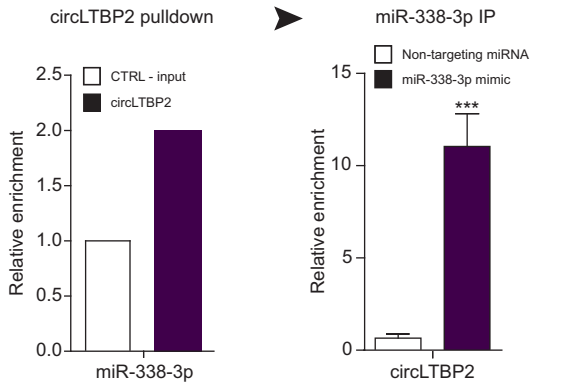
(10 μg/ml) to inhibit cell proliferation. (E) Resistance of HuCC-T1 cells to gemcitabine-induced apoptosis (10 ng/ml) was evaluated every 4 h using a red fluorescent Annexin V labelling, in both the circLTBP2 overexpressing and control groups. (F) Mouse subcutaneous tumour model was used to evaluate the impact of circLTBP2 overexpression on tumour growth (G, H) and weight (I). Five mice were included in the control group, and 6 mice were included in the circLTBP2 overexpression group. Statistical analyses for (B) and (I) were performed using a Mann-Whitney *U* test. Statistical analyses for (C–E), and (H) were performed using a two-way ANOVA. *p <0.05, **p <0.01, ***p <0.001, n ≥3 technical and biological replicates. CCA, cholangiocarcinoma; circRNA, circular RNA; iCCA, intrahepatic CCA; pLV-circLTBP2, circLTBP2 overexpression vector; pLV-circRNA, empty vector; LTBP2, latent TGFβ-binding protein 2; TGFβ, transforming growth factor β.

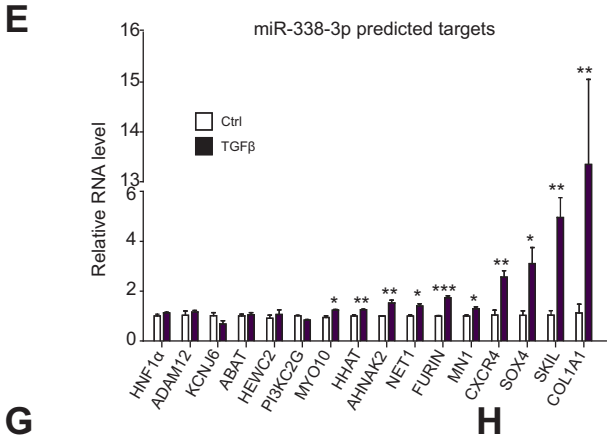


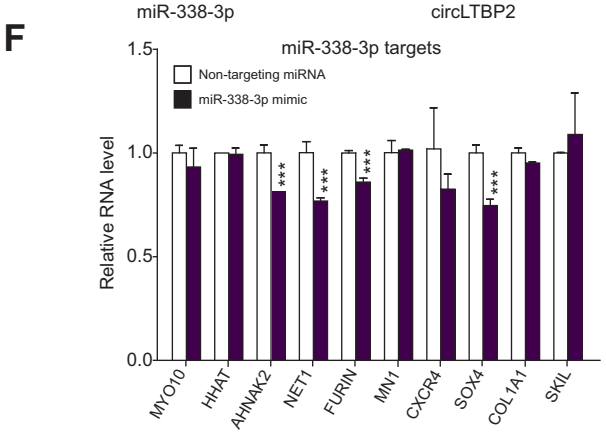
B

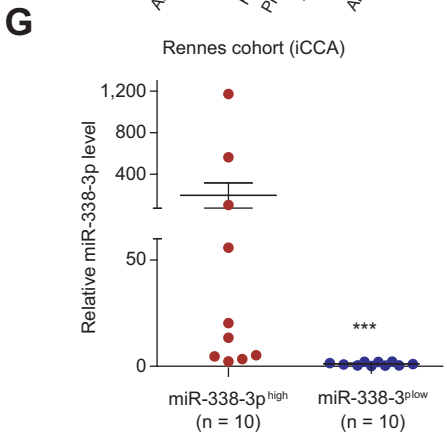
miRNA ID	circLTBP2 binding sites	GSEA NES score	Relavance rank
miR-338-3p	7	1.86	1
miR-3064-5p	3	1.37	2
miR-1270	3	1.36	3
miR-1294	3	1.30	4
miR-34c-5p	2	1.59	5
miR-324-3p	2	1.68	6
miR-1914-3p	2	1.69	7
miR-34a-5p	2	1.64	8











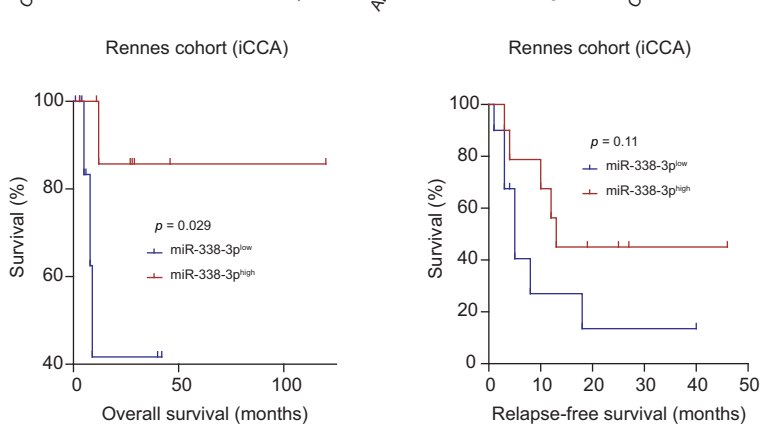


Fig 3. CircLTBP2 acts as a sponge for miR-338-3p, a known tumour suppressor miRNA. (A) Schematic representation of the strategy used to select a miRNA showing strong interactions with circLTBP2, based on the data obtained by pulldown assay followed by a miRNA sequencing. (B) Relevance rank of circLTBP2 binding miRNAs based on the predicted number of binding sites (https://starbase.sysu.edu.cn/) and the GSE score of their mRNA targets in the profile of HuCC-T1 cells treated with TGFβ (GSE102109). (C) GSE score of miR-338-3p mRNA targets in HuCC-T1 TGFβ signature (GSE102109). The top 20 enriched genes are listed. (D) RNA IP of AGO2 in HuCC-T1 cells transfected with miR-338-3p mimics and quantification by q-RT-PCR of putative miR-338-3p binding RNA (*i.e.* circLTBP2). (E) Expression of miR-338-3p targets after TGFβ treatment evaluated by q-RT-PCR. (F) Expression of miR-338-3p targets evaluated by q-RT-PCR in HuCC-T1 cells

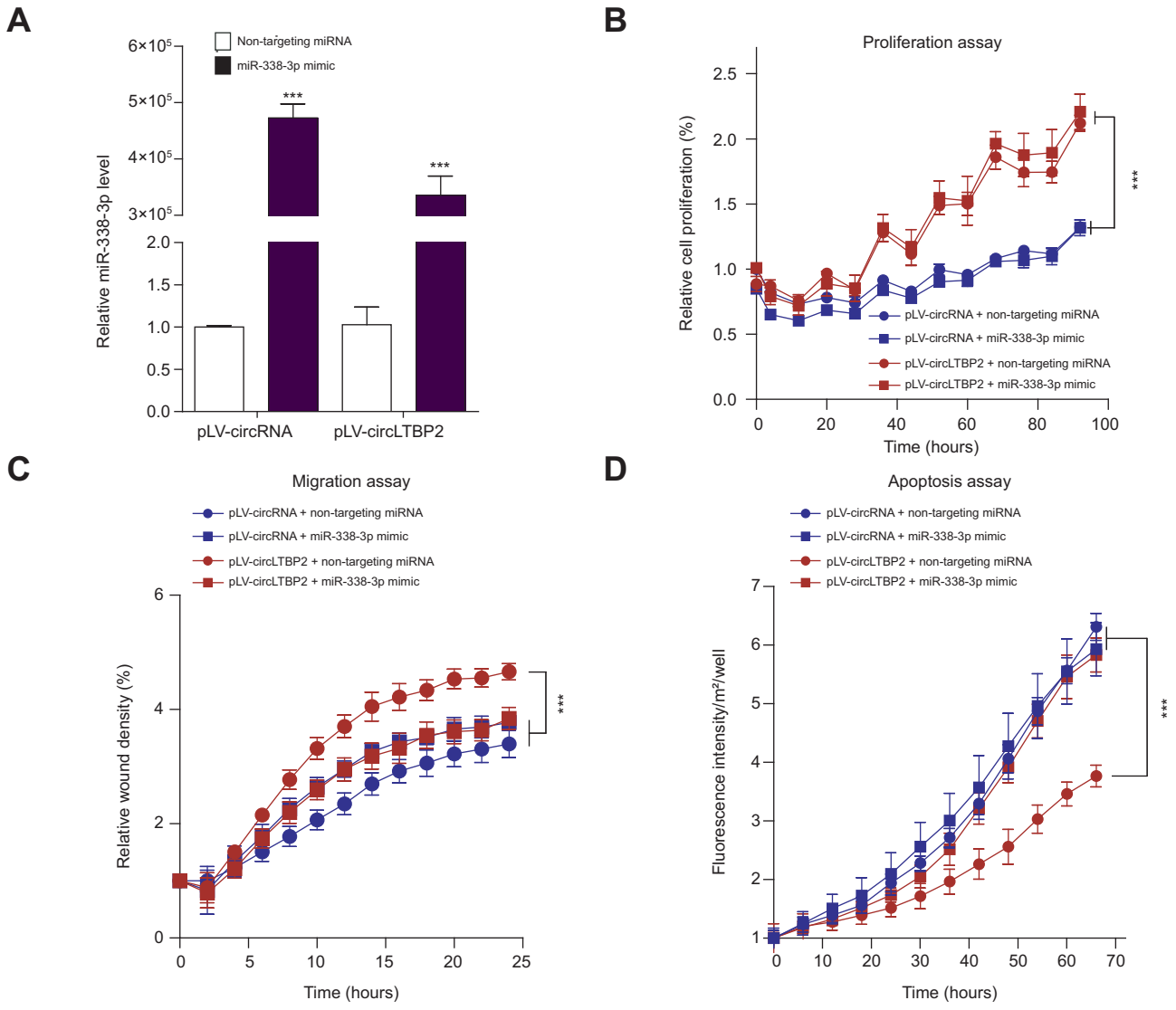


Fig. 4. The restoration of miR-338-3p levels in iCCA cells reversed the pro-tumourigenic effects driven by circLTBP2. (A) Relative levels of miR-338-3p in HuCC-T1 were assessed by q-RT-PCR in cells overexpressing circLTBP2 *vs*. cells infected with the empty vector. In addition, miR-338-3p mimics (5 nM) were added with the aim of rescuing the sponging mediated by circLTBP2. (B) Relative confluency of cultured HuCC-T1 cells was evaluated for 96 h in HuCC-T1 cells overexpressing circLTBP2 compared with the empty vector control group (n = 3 per group). In addition, miR-338-3p mimics were added aiming at restoring the phenotype. (C) Cell migration was evaluated by wound healing assay performed on HuCC-T1 cells overexpressing circLTBP2 compared with the empty vector control group (n = 3 per group). In addition, miR-338-3p mimics (5 nM) were added aiming at restoring the phenotype. (D) Resistance of HuCC-T1 cells to gemcitabine-induced apoptosis (10 ng/ml) was evaluated every 4 h using a red fluorescent Annexin V labelling, in both the circLTBP2 overexpressing and control groups. In addition, miR-338-3p mimics were added aiming at restoring the phenotype (5 nM). Statistical analyses for (A) were performed using a Mann-Whitney *U* test. Statistical analyses for (B–D) were performed using a two-way ANOVA. **p* <0.05, ***p* <0.01, ****p* <0.001, n ≥3 technical and biological replicates. CCA, cholangiocarcinoma; circRNA, circular RNA; iCCA, intrahepatic CCA; pLV-circLTBP2, circLTBP2 overexpression vector; pLV-circRNA, empty vector; LTBP2, latent TGFβ-binding protein 2; q-RT-PCR, quantitative reverse-transcription PCR; TGFβ, transforming growth factor β.

sites (>2) and the enrichment score (>1.3) of the predicted miRNA targets in the gene expression profile of HuCC-T1 cells stimulated by TGFβ. In total, eight miRNAs met the selection criteria (miR-338-3p, miR-3064-5p, miR1270, miR-1294, miR-34c-5p, miR324-3p, miR-1914-3p, and miR-34a-5p) (Fig. 3B). Among them, miR-338-3p had the highest number of predicted binding sites (n = 7) on circLTBP2 (Fig. 3B), and the highest enrichment score (normal enrichment score = 1.86, *p*

transfected with miR-338-3p mimics (5 nM). (G) Mir-338-3p relative expression in 20 freshly frozen iCCA tumours from a French national cohort (separated in two groups, namely, miR-338-3p^{low} and miR-338-3p^{high}, according to their median expression). (H) Kaplan-Meier plots and log-rank statistics analysis of overall and relapse-free survival for patients with iCCA with high *vs*. low expression of miR-338-3p. Statistical analyses for (D–G) were performed using a Mann-Whitney *U* test. **p* <0.05, ***p* <0.01, ****p* <0.001, n ≥3 technical and biological replicates. CCA, cholangiocarcinoma; GSEA, gene set enrichment analysis; iCCA, intrahepatic CCA; IP, immunoprecipitation; LTBP2, latent TGFβ-binding protein 2; miRNA, microRNA; NES, normal enrichment score; q-RT-PCR, quantitative reverse-transcription PCR; TGFβ, transforming growth factor β.

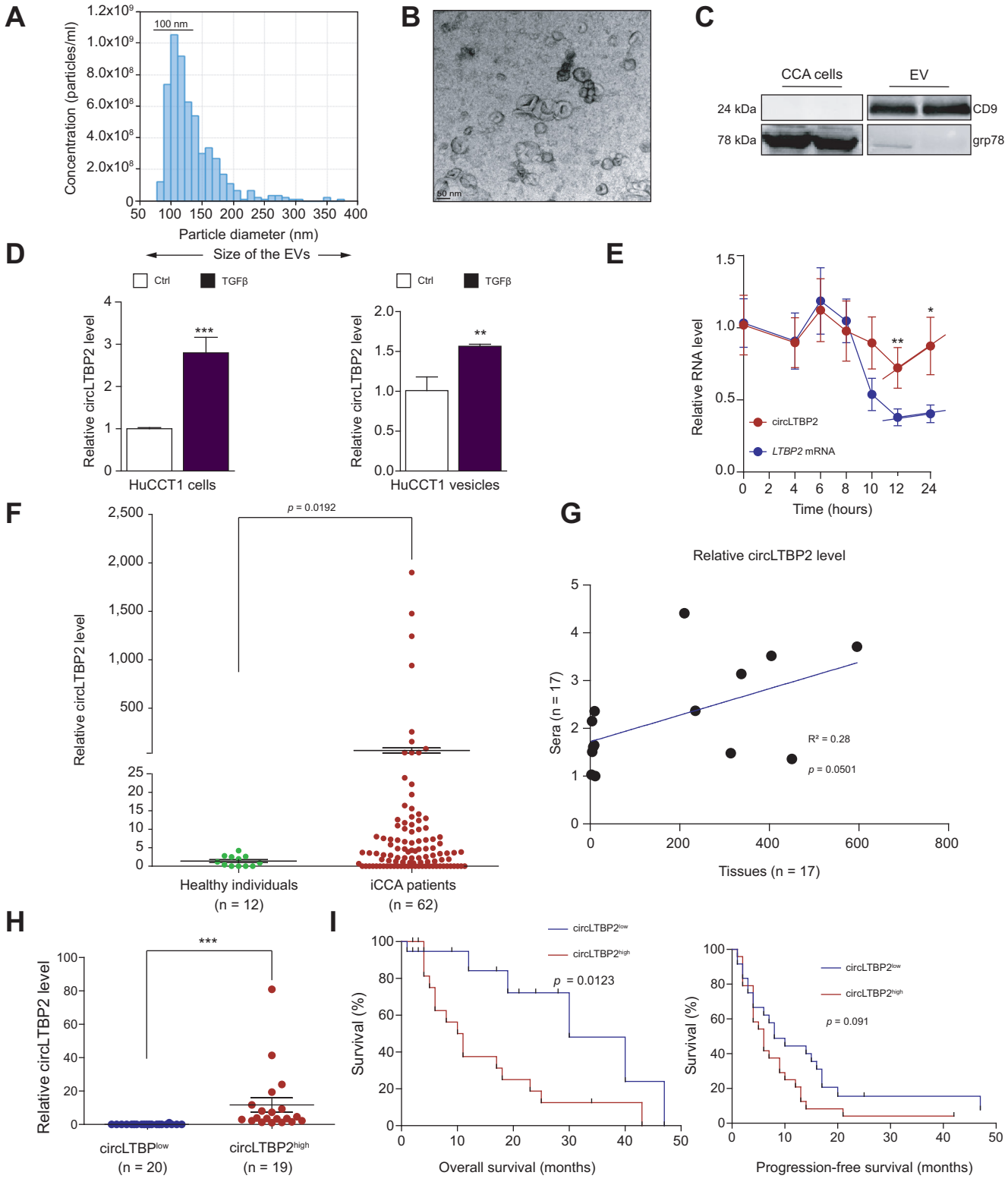


Fig. 5. Circulating vesicles containing circLTBP2 in serum are associated with poor OS in patients with iCCA. (A) Size and concentration of EVs collected from HuCC-T1 supernatant by UC were assessed using a tunable resistive sensing method (qNano, Izon Science). EVs with a size ranging from 100 to 200 nm were characterised. (B) Scanning electron microscopy was used to image EVs collected after UC. The morphology and size observed match those of EVs. (C) Immunoblotting of EV marker CD9 in HuCC-T1 cells and EVs isolated from their supernatant. The protein GRP78 was used as a cell specific negative control. (D) Relative levels of circLTBP2 in HuCC-T1 cells and EVs isolated from their supernatant by UC. HuCC-T1 cells were first treated with TGFβ (1 ng/ml, 16 h). (E) Half-life of circLTBP2 in HuCC-T1 cells treated with actinomycin D (2 μg/ml) for 24 h. (F) Quantification by q-RT-PCR of EV-embed circLTBP2 in sera from patients with metastatic advanced iCCA (Rennes cohort, n = 62). Sera from healthy individuals were used as control (n = 12). (G) Positive correlation of circLTBP2 expression in matched tissues and serum samples (patients for which both samples are available, n = 17). (H) EV-embed circLTBP2 relative expression in sera from patients with

<0.01), as determined by gene set enrichment analysis (Fig. 3C and Fig. S9). To confirm the pulldown assay, we performed a miRNA IP assay that demonstrated the direct interaction between miR-338-3p and circLTBP2 (Fig. 3D). Interaction with linear LTBP2 was also highlighted (Fig. S10), suggesting that miR-338-3p could also be sponged by TGFβ-induced LTBP2 mRNA. However, functional tests demonstrated that linear LTBP2 did not significantly impact the behaviour of HuCCT-1 cells, suggesting that the observed effects were specific from circLTBP2 (Fig. S11). Among 16 miR-338-3p targets possibly involved in biological processes regulated by circLTBP2, the expression of 10 was significantly increased by TGFβ (Fig. 3E). Four of these targets (*SOX4*, *ANHAK2*, *FURIN*, and *NET1*) also exhibited a small but significant decrease in expression in cells transfected with miR-338-3p mimics (Fig. 3F). RNA IP further demonstrated a direct interaction between miR-338-3p and SOX4 (Fig. S10). Mirroring the results observed with circLTBP2 (Fig. 1F and G), a reduced expression of miR-338-3p in human CCA tumours was associated with a significant reduced OS (p <0.05) (Fig. 3G and H). However, no statistically significant anticorrelation between circLTBP2 and miR-338-3p levels was observed. Next, to determine whether the pro-tumourigenic effects driven by circLTBP2 resulted from the sequestration of miR-338-3p, we conducted rescue experiments using miRNA mimics. Importantly, circLTBP2 expression does not affect the expression of miR-338-3p at the basal level (*i.e.* without miRNA mimics) (Fig. 4A). Although described as a tumour suppressor miRNA, miR-338-3p alone does not significantly impact cell proliferation, migration, and apoptosis (Fig. 4B–D). Restoring miR-338-3p had no impact on circLTBP2-induced cell proliferation (Fig. 4B), suggesting that additional mechanisms are involved. Because circRNA-encoded peptides may drive the function of circRNAs, we evaluated the coding potential of circLTBP2. Prediction algorithms and gene expression analysis of actively translated RNAs demonstrated that circLTBP2 was unlikely translated (Fig. S12). Interestingly, rescuing miR-338-3p levels was able to decrease circLTBP2-induced cell migration and to sensitise cells to gemcitabine-induced apoptosis (Fig. 4C and D).

Circulating circLTBP2 is associated with poor OS in patients with iCCA

Finally, we aimed at evaluating the clinical relevance of circLTBP2 as a prognostic biomarker for iCCA, easily detectable by non-invasive methods. Thus, we first determined whether circLTBP2 could be embedded into extracellular vesicles (EVs) and secreted. EVs (mainly exosomes) were isolated by ultracentrifugation and qualified by qNano (Izon Science), transmission electron microscopy, and expression of specific surface markers (Fig. 5A–C). Interestingly, circLTBP2 was expressed in EVs secreted by HuCCT-1 cells, and its expression in EVs was greater in cells pretreated by TGFβ (Fig. 5D). In addition, circLTBP2 was more stable than its linear counterpart with a half-life greater than 24 h (Fig. 5E). More interestingly, the expression level of circLTBP2 was significantly increased in EVs isolated from the serum of patients with advanced, metastatic iCCA (n = 62) *vs.* healthy individuals (Fig. 5F). A significant correlation between the levels of circLTBP2 in the tumour tissues and serum was also highlighted (Fig. 5G). From patients with available survival data (n = 39), we generated two groups based on the median expression of circLTBP2 (Fig. 5H). Statistical analysis demonstrated that a high expression of circulating circLTBP2 was significantly associated with a reduced OS (Fig. 5I).

Discussion

In this study, we identified circRNAs modulated by TGFβ in iCCA cells. In addition, we investigated the role and the underlying molecular mechanisms by which TGFβ-induced circLTBP2 modulates iCCA progression. We demonstrated that an increased expression of circLTBP2 promotes iCCA cell proliferation and migration, as well as resistance to gemcitabine-induced apoptosis. Mechanistically, the data suggest that circLTBP2 exerts some of its pro-oncogenic actions by modulating the activity of miR-338-3p, a well-known tumour suppressor miRNA.[16] We also report that circLTBP2 expression in tissues and sera from patients with iCCA was associated with a worse prognosis.

Only a few studies have reported in depth functional studies of circRNAs involved in iCCA oncogenesis so far.[8,9] For example, circACTN4 acts as a signalling nexus allowing for the coordinated activation of Hippo and Wnt pathways. Mechanistically, circACTN4 is able to sponge miR-424-5p in the cytoplasm, allowing for the expression of Yes-associated protein 1 (YAP1) and to recruit YBX1 at the promoter of *FZD7* in the nucleus[10,12] CircRNA MBOAT2 promotes iCCA progression and lipid metabolism reprogramming by stabilising PTBP1 to facilitate FASN mRNA cytoplasmic export.[17] Interestingly, although initially classified as non-coding RNAs, some circRNAs have been shown to be translated. Thus, the protein encoded by IL-6-induced circGGNBP2 was shown to promote CCA cell growth and metastasis, notably through a positive regulatory loop modulating IL-6/STAT3 signalling.[11] Our experimental data suggest that circLTBP2 is unlikely to be translated into functional peptides. Secreted circRNAs could also play a key role in CCA carcinogenesis, as illustrated by circ_0020256 in exosomes from tumour-associated M2 macrophages, which has been shown to promote CCA cell proliferation, migration, and invasion by modulating a miR-432-5p/E2F3 axis.[18]

LTBP2 belongs to the family of LTBP proteins involved notably in the bioavailability of TGFβ in the tumour microenvironment. However, LTBP2 is unique in the family as it does not bind to latent TGFβ. LTBP2 associates with the extracellular matrix, and it has been suggested that it may indirectly regulate the activation of TGFβ by releasing LTBP1 from microfibrils.[19] Increased expression of LTBP2 has been reported in several cancers and associated with tumour progression, notably in HCC.[20] Here, we provide the first evidence of the induction of its circular counterpart by TGFβ in CCA. Interestingly, circLTBP2 overexpression does not affect the expression of LTBP2, as it could have been

iCCA whose survival data were available (separated in two groups, namely, circLTBP2^{low} and circLTBP2^{high}, according to their circLTBP2 median expression). (I) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased OS for patients with iCCA with a high expression of vesicles-embed circLTBP2. Levels of circLTBP2 were not associated with the RFS. Statistical analyses for (A) were performed using a Mann-Whitney *U* test. Statistical analyses for (B–D) were performed using a two-way ANOVA. *p <0.05, **p <0.01, ***p <0.001, n ≥3 technical and biological replicates. CCA, cholangiocarcinoma; EV, extracellular vesicle; iCCA, intrahepatic CCA; LTBP2, latent TGFβ-binding protein 2; OS, overall survival; pLV-circLTBP2, circLTBP2 overexpression vector; pLV-circRNA, empty vector; q-RT-PCR, quantitative reverse-transcription PCR; RFS, relapse-free survival; TGFβ, transforming growth factor β; UC, ultracentrifugation.

reported for some circRNAs, suggesting that the effects observed in GOF/LOF experiments are directly related to circLTBP2. We showed that circLTBP2 acts as a sponge for miR-338-3p and thus may protect its pro-oncogenic targets from degradation. Because both linear and circular LTBP2 are regulated by TGFβ and both harbour binding sites for miR-338-3p, it is possible that both transcripts sponge and modulate the activity of miR-388-3p. However, we hypothesise that the different stability of linear *vs*. circular LTBP2 transcripts may greatly influence the modulation of miRNA activity. Indeed, our data demonstrated a greater half-life for circLTBP2, suggesting a more potent sponging activity. In addition, functional tests demonstrated that linear LTBP2 did not significantly impact on CCA cell behaviour, suggesting that the observed effect was specific from circLTBP2. However, additional investigations will be required to strengthen the involvement of miR-338-3p, as, for example, by mutating its binding sites on circLTBP2. MiR-338-3p plays a crucial role in tumour progression, and its activity is tightly regulated in cancer. It exhibits a strong prognostic value for biliary tract cancers, and low levels of miR-338-3p have been measured in advanced-stage CCA associated with lymph node infiltration. Notably, it has been identified in a three-miRNAs signature that predicts survival in patients with iCCA. Patients with low levels of miR-338-3p had the worse OS and RFS.[21] Many pathways depend on the activity of miR-338-3p, including WNT, MAPK, and PI3K/AKT, all of which are involved in the progression of iCCA.[22] Accordingly, miR-338-3p controls numerous hallmarks of cancer cells, such as inhibiting cell proliferation signals, inducing cell death, or decreasing angiogenesis.[16] Our rescue experiments demonstrated that the circLTBP2/miR-338-3p axis regulates cell migration and resistance to gemcitabine-induced apoptosis. Our data suggest that miR-338-3p possibly targets SOX4, a key gene involved in epithelial-to-mesenchymal transition, modulating master regulators TWIST1, SNAI1 and ZEB1.[23] The regulation of SOX4 by miR-338-3p has already been described in several cancers (*e.g.* breast cancer and kidney cancer) and associated with cell proliferation and migration.[24] In lung cancer, miR-338-3p has been shown to suppress metastasis by targeting SOX4.[25] However, restoring miR-338-3p had no impact on circLTBP2-induced cell proliferation, suggesting that additional mechanisms are involved. Another puzzling result is the different action of TGFβ and TGFβ-induced circLTBP2 on the proliferation of HuCCT-1 cells. Indeed, although both TGFβ and circLTBP2 promoted cell migration and resistance to apoptosis, they inhibited and promoted cell proliferation, respectively. Thus, cirLTBP2 could act as an effector of the pro-tumourigenic responses of TGFβ associated with cell migration and resistance to apoptosis, but as a negative feedback regulator to counteract the cytostatic effects of TGFβ in HuCCT-1 cells. Even though a similar observation on cell proliferation was made in TFK1 cells, additional investigation will be required to determine the specificity of these responses in other CCA cell lines.

Although further validation using large cohorts of patients will be needed to confirm the link between circLTBP2 expression and survival, our data highlight the clinical relevance of circLTBP2 as a prognostic biomarker in three independent cohorts of iCCA and HCC. The diagnosis of some iCCA may represent a clinical challenge owing to anatomo-morphological similarities between tumours developing along the hepatobiliary tree.[26] In this study, we report a differential expression of circLTBP2 between iCCA and HCC, suggesting that circLTBP2 could be relevant to differentiate these two types of tumours. CCA comprises a heterogeneous group of tumours and applying a systematic and effective therapeutic approach for all patients is challenging. The current first-line reference treatment is a combination of gemcitabine and cisplatin. However, the benefit of this treatment is modest, and iCCA remains a poor prognosis cancer.[27–29] Our data further suggest that inhibiting circLTBP2 (*e.g.* by using antisense oligonucleotides) could be relevant to sensitise iCCA cells to gemcitabine. Translational studies supporting this point will be required.

In summary, our study demonstrates that circRNAs modulate iCCA carcinogenesis and may serve as a clinically relevant predictive biomarker for a better management of patients. In addition, the study provides evidence for the regulatory role of the TGFβ/circLTBP2/miR-338-3p axis in iCCA. These findings provide a basis for further exploration of circRNAs in iCCA, including their function, and diagnostic and prognostic therapeutic potential.

Abbreviations

CCA, cholangiocarcinoma; CDH1, E-cadherin; circRNA, circular RNA; EV, extracellular vesicle; GOF, gain-of-function; HCC, hepatocellular carcinoma; iCCA, intrahepatic CCA; IP, immunoprecipitation; LOF, loss-of-function; LTBP2, latent TGFβ-binding protein 2; miRNA, microRNA; OS, overall survival; q-RT-PCR, quantitative reverse-transcription PCR; RFS, relapse-free survival; TGFβ, transforming growth factor β; TGFBR1, TGFβ receptor type 1; VIM, vimentin; YAP1, yes-associated protein 1.

Financial support

This work was supported by Inserm, Université de Rennes 1, CLCC Eugène Marquis, and ITMO Cancer AVIESAN (Alliance Nationale pour les Sciences de la Vie et de la Santé) dans le cadre du Plan cancer (Non-coding RNA in cancerology: fundamental to translational), Ligue contre le cancer (CD22, CD29, CD35, CD44, CD49, CD85), and Ligue nationale contre le cancer, and received financial support from ITMO Cancer of AVIESAN within the framework of the 2021–2030 Cancer Control Strategy, on funds administered by Inserm. This work has been supported by the Fondation ARC pour la recherche sur le cancer and has received grants from the French Ministry of Health and the French National Cancer Institute, PRTK20-136, PLBIO21-212, CHU Rennes, CLCC Eugène Marquis, Rennes. JV was funded by the Spanish Ministry for Science and Innovation (MCIN: 'Ramon y Cajal' program RYC2021-034121-I). CC, JE, JV, and RIRM are members of the European Network for the Study of Cholangiocarcinoma (ENSCCA) and participate in the initiative COST Action EURO-CHOLANGIO-NET granted by the COST Association (CA18122).

Conflict of interest

JE received honoraria from MSD, Roche, AstraZeneca, and BMS. The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership, or options, expert testimony, grants or patents received or pending, or royalties.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived the project: CC. Provided funding and supervision: CC, JE. Designed the experiments: CC, JE, CL. Acquired the data: CL, TF, RL, RP, MD, PP, GA, DL, JV. Provided samples: RIRM. Analysed the data: CC, CL, MD, RP, DL. Interpreted the data: CC, JE, CL, MD, RP. Critically revised the article: all authors.

Data availability statement
Data sharing is subject to agreement.

Acknowledgements
The authors thank E. Dumontet, S. Dréano, and C. Pellegrinelli for their technical help. The authors thank L. Fouassier for providing CCA cell lines, V. Quillien for providing sera, and C.-H. Heldin for providing linear LTBP2 expression plasmid. The authors thank the core facilities of Biosit and Biogenouest.

Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100900.

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Author names in bold designate shared co-first authorship

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