The long noncoding RNA IncPARP1 contributes to progression of hepatocellular carcinoma through up-regulation of PARP1

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Hepatocellular carcinoma (HCC) accounts for a large proportion of cancer-associated mortality worldwide. The functional impact of long noncoding RNAs (lncRNAs) in human cancer is not fully understood. Here, we identified a novel oncogenic lncRNA termed as IncPARP1, which was significantly up-regulated in HCC. Increase in IncPARP1 expression was associated with age, α-fetoprotein (AFP) levels, tumor size, recurrence, and poor prognosis of HCC patients. Loss-of-function approaches showed that knockdown of IncPARP1 inhibited proliferation, migration, and invasion, while induced apoptosis in HCC cells. Moreover, mechanistic investigation demonstrated that PARP1 was an underlying target of IncPARP1 in HCC. In summary, we provide the first evidence that IncPARP1 exerts an oncogene to promote HCC development and progression, at least in part, by affecting poly (ADP-ribose) (PAR) polymerase 1 (PARP1) expression.

Introduction

As one of the most common cancers, hepatocellular carcinoma (HCC) accounts for a large proportion of cancer-associated mortality worldwide, which causes more than 600000 deaths each year [1,2]. In spite of the remarkable improvements in comprehensive HCC treatment, such as surgery, chemotherapy, radiotherapy, and targeted therapy, the prognosis of patients with HCC remains unsatisfactory. Therefore, it is extremely urgent to reveal the molecular mechanisms in the course of HCC development, which will be greatly helpful to identify novel diagnostic and therapeutic markers for HCC patients.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs with larger than 200 nts in length but lack the significant protein-coding capacity. Initially, IncRNA was identified as the by-product of transcription by RNA polymerase and without biological function [3]. However, emerging evidence demonstrated that lncRNAs play crucial roles in regulating gene expression via diverse mechanisms, including epigenetic alteration, transcriptional control, and post-transcriptional modification [4,5]. Dysregulation of lncRNAs associate with HCC initiation and progression [6,7]. For instance, lincRNA-UFC1 was overexpressed in HCC tissues, and associated with tumor size, clinical stage, and patient prognosis. LincRNA-UFC1 promoted proliferation and cell cycle progression and inhibited apoptosis through interacting with the mRNA stabilizing protein HuR and enhancing β-catenin expression [8]. Although thousands of lncRNAs have been annotated, only a few lncRNAs have been functionally characterized.

Poly (ADP-ribose) (PAR) polymerase 1 (PARP1) is a DNA-dependent ADP-ribosylation transferase, which regulates protein ADP-ribosylation and the functions of the modified proteins. PARP1 has been
Expression of IncPARP1 is up-regulated in HCC, which is clinically correlated with the poor prognosis of patients with HCC.

In our previous study, we analyzed the differential expression of IncRNAs and mRNAs between HCC and adjacent non-tumor tissues through microarray detection [18]. Here, amongst these up-regulated IncRNAs, we screened out a novel functional IncRNA (GeneSymbol: BC032899) named as IncPARP1. We then characterized the pathological relationship of IncPARP1 in HCC growth and progression. Further investigation showed that IncPARP1 functions as an oncogenic IncRNA to regulate proliferation, migration, and invasion via modulating PARP1 expression.

Materials and methods

Patients and tissue samples
The 70 pairs of HCC and corresponding non-tumor tissues were collected from patients with HCC who initially underwent hepatectomy without any preoperative treatment at the Zhongshan Hospital of Xiamen University from 2013 to 2016. The procedure for sample collection was approved by the ethics committee of the Zhongshan Hospital of Xiamen University, and written informed consent was obtained from all the patients.
Figure 2. IncPARP1 regulates HCC cell proliferation and apoptosis in vitro

(A) Relative IncPARP1 expression in Bel-7402 and PLC/PRF/5 cells transduced with lentivirus expressing control shRNA (con), IncPARP1 shRNA1 (sh1), shRNA2 (sh2), or shRNA3 (sh3) was determined by performing qRT-PCR. (B) Proliferation of control and IncPARP1-knockdown cells was assessed by performing the CCK-8 assay. (C) Colony formation assays were performed using control and IncPARP1-knockdown cells. (D) IncPARP1-knockdown cells were treated with 5-fluorouracil (100 mg/ml) for 48 h, stained with Annexin V and propidium iodide (PI), and analyzed by flow cytometry. Annexin V-positive cells were designated as apoptotic cells. Percentage of apoptotic cells is shown. (E) The expression of caspase-3 and cleaved caspase-3 in Bel-7402 cells with IncPARP1 knockdown detected by Western blot. *P<0.05, **P<0.01.
Figure 3. IncPARP1 enhances HCC migration and invasion
Left, representative images of the migration and invasion of Bel-7402 and PLC/PRF/5 cells expressing control and IncPARP1 shRNAs. Right, statistical results obtained from three independent experiments. **P < 0.01.

Ethics approval and consent to participate
All protocols dealing with the patients conformed to the ethical guidelines of the Helsinki Declaration and were approved by the Medical Ethics Committee of Hospital of Zhongshan Hospital of Xiamen University.

Cell culture
SMMC-7721, HepG2, HuH7, SK-Hep-1, PLC/PRF/5, and Bel-7402 cells were obtained from the Cell Bank of Chinese Academy of Sciences, and MHCC-97h cells were obtained from Zhongshan Hospital of Fudan University. Cells were cultured in DMEM (HyClone) supplemented with 10% (FBS; Gibco) at 37°C. Authentication of these cell lines was performed using the GenePrint 10 System (Promega) and via comparisons with the STR database.

RNA extraction and quantitative real-time PCR
Total RNA was isolated by TRIzol reagent (Invitrogen) as per manufacturer’s instructions. cDNA was synthesized using One-Step gDNA Removal and cDNA Synthesis Kit (Transgen, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed in the Lightcycler 96 Real-Time PCR System (Roche) using FastStart Universal SYBR Green Master (Roxy) (Roche). The gene-specific primers are shown in Supplementary Table S1. ACTB was employed as an endogenous control. Comparative quantitation was determined using the 2^(-ΔΔC_t) method.

Construction of stable cells with IncPARP1 knockdown
Three different shRNAs targeting IncPARP1 were inserted into the pLV-shRNA-puro plasmid (Invogen Tech, Beijing, China). The target sequences of IncPARP1 shRNAs are shown as follows: sh1: CCGCTGACAGAAAGGAACCTT, sh2: CAGGAGATGGGAGCAACAA, and sh3: CGCCTCCAGATTGAACCTGTCT. The stable clones expressing scramble shRNA were taken as control and the sequence was: CCTAAGGTAAATCGCCCTCAGTCGAGGC-GGAGCCACTTACCTTAGG. Lentiviral expressing and packaging vectors were transfected into HEK-293T cells by using TurboFect Transfection Reagent (Thermo Scientific) according to the manufacturer’s instructions. Then the medium containing the lentivirus was harvested and transduced into HCC cells in the presence of 5 μg/ml polybrene (Sigma). Stable cells were selected using puromycin for 1 week.

Cell proliferation and colony formation assay
For cell proliferation assay, 3 × 10^3 cells per well were seeded in 96-well culture plates. At the indicated time points, CCK-8 (Dojindo) was added to each well and incubated at 37°C for 1.5 h. The absorbance values were measured using a microplate reader (Bio–Rad) at 450 nm wave length. For colony formation assay, 3 × 10^3 cells per well were
seeded in the six-well culture plates. After 14 days’ culture, cells were fixed with 4% paraformaldehyde and stained with Crystal Violet.

**Apoptosis analysis**
The apoptosis was detected by using Apoptosis Detection Kit (Dojindo) according to the manufacturer’s instructions. The data were analyzed by Kaluza software.

**Migration and invasion assay**
HCC cells (3 × 10^5) were suspended and seeded in 200 μl serum-free DMEM in the upper chamber of a 24-well Transwell migration (Corning) or invasion insert (BD Biosciences). The lower chamber was filled with DMEM containing 10% FBS. After 24 h of incubation, the cells that had traversed the membrane were fixed in 4% paraformaldehyde and then staining by Crystal Violet for 30 mins.

**Western blot**
Cells or tumor tissue samples were lysed with RIPA buffer (Beyotime) containing protease inhibitors cocktail (Sel- leck). After centrifugation, samples were loaded on to and separated using SDS/PAGE, then transferred on to PVDF membranes (Millipore). The membranes were blocked for 1 h at room temperature and incubated with the anti-β-actin (#3700, Cell Signaling Technology), anti-PARP1 (13371-1-AP, Proteintech) and anti-Caspase 3 (#9662, Cell Signaling Technology) antibodies overnight at 4°C. After washing, the blots were incubated with goat anti-rabbit (111-035-003, Jackson) or anti-mouse (115-035-003, Jackson) HRP-conjugated secondary antibodies and visualized using the Immobilon™ HRP Substrate Peroxide Solution (Millipore).

**Xenograft assay**
A xenograft mouse model was developed using 5–6-week-old male BALB/c nude mice. HCC cells (5 × 10^6) were injected subcutaneously into the right flank of the nude mice. Tumors were detected after ~14 days, and tumor size was measured every 3 days. Tumor-bearing mice were killed 38 days after the injection, and tumors were removed for further analysis.

**Immunohistochemical**
Four micrometer sections were pretreated before immunohistochemical (IHC) staining. After deparaffinization and an antigen retrieval step, endogenous peroxidase was quenched by incubation of the sections with 0.3% hydrogen peroxide for 15 min at room temperature. The sections were blocked by non-immune serum for 15 min at room temperature and then incubated with anti-PARP1 antibody (1:200, Proteintech, 13371-1-AP) or proliferating cell nuclear antigen (PCNA) antibody (1:200, Proteintech, 10205-2-AP) at 4°C overnight. After washing, the sections were incubated with biotinylated secondary antibody (KIT-5010, Maixin Biotechnology, Fuzhou, China), and then visualized using dianaminobenzidine (Maixin Biotechnology, Fuzhou, China).

**Statistical analysis**
All experiments were performed in triplicate. Statistical analyses were performed by using the SPSS software (version 19.0). Data are shown as mean ± S.E.M. The differences between groups were analyzed by the Student’s t test or chi-square test. The Kaplan–Meier method was performed for patients’ overall survival analysis. P<0.05 was considered statistically significant difference.

**Results**

**IncPARP1 is overexpressed in HCC tissues and correlates with poor prognosis of HCC patients**
First, we analyzed the differential expression of lncRNA and mRNA between HCC and adjacent non-tumor tissues through microarray detection from our previous study [18]. We focussed on the tumoral up-regulated lncRNAs nearby cancer-related protein coding genes and found an overexpressed lncRNA (GeneSymbol: BC032899) named IncPARP1, which was located downstream of PARP1 gene. We then performed qRT-PCR to examine Inc- PARP1 expression levels in 70 pairs of HCC and corresponding adjacent non-tumor tissue samples (Figure 1A). IncPARP1 levels were significantly increased in HCC tissues compared with that in the non-tumor tissues. To clarify the clinical significance of IncPARP1, we analyzed the correlation between IncPARP1 expression and clinicopathologic features of HCC patients. Intriguingly, IncPARP1 up-regulation was markedly associated with elder age,
Figure 4. The correlation between PARP1 and lncPARP1 expression in HCC

(A, B) PARP1 mRNA (A) and protein expression (B) in control and lncPARP1 knockdown HCC cells was analyzed by performing qRT-PCR and Western blot, respectively. (C) PARP1 mRNA expression in 70 pairs of HCC and corresponding non-tumor tissues was determined by performing qRT-PCR. (D) PARP1 protein expression in 18 pairs of HCC and peritumor tissues were analyzed by Western blot. (E) The correlation between PARP1 and lncPARP1 in HCC tissue samples. *P<0.05.

higher level of serum α-fetoprotein (AFP), larger tumor size, and recurrence. No significant correlation between lncPARP1 expression and other factors was observed, such as gender, differentiation, and invasion (Table 1). Moreover, Kaplan–Meier and log-rank test analyses suggested a significant correlation between the lncPARP1 expression and dramatically decreased overall survival and tumor-free survival rates (Figure 1B,C). Above all, these results indicate that up-regulation of lncPARP1 may be involved in HCC progression.

**lncPARP1 promotes proliferation and inhibits apoptosis in HCC cells**

We then investigated the functional roles of lncPARP1 in HCC progression. To choose the HCC cell lines used for loss-of-function assays, we detected the lncPARP1 expression in seven different HCC cell lines, including Bel-7402, SMMC-7721, MHCC-97h, HepG2, Huh7, PLC/PRF/5, and SK-Hep-1 cells. Amongst these HCC cells, Bel-7402 and PLC/PRF/5 cells expressed the highest level of lncPARP1 (Supplementary Figure S1). Therefore, Bel-7402 and PLC/PRF/5 cells were selected for knockdown of lncPARP1. To avoid off-target effects, we designed three independent shRNAs against lncPARP1, and the two most efficient (sh2 and sh3) were selected for further experiments (Figure 2A). To analyze the effects of lncPARP1 silence on HCC cell proliferation, we performed CCK-8 assay and...
found that knockdown of lncPARP1 remarkably inhibited proliferation of both Bel-7402 and PLC/PRF/5 cells, compared with that of control cells (Figure 2B). Similarly, lncPARP1 inhibition by shRNAs significantly suppressed the colony-forming ability of HCC cells (Figure 2C).

To further determine mechanisms underlying the lncPARP1-mediated cell proliferation, we checked the influence of lncPARP1 knockdown on cell cycle distribution by performing flow cytometry. However, the results illustrated that lncPARP1 silence did not affect cell cycle progression, suggested that lncPARP1 did not modulate cell cycle (Supplementary Figure S2). Given that lncPARP1 functions as an oncogenic lncRNA in HCC cells, we speculated that lncPARP1 may be critical for cell survival and apoptosis. To verify this hypothesis, we analyzed the apoptosis by performing flow cytometry in HCC cells with Annexin V and propidium iodide (PI) staining. As shown in Figure 2D, lncPARP1-silencing Bel-7402 and PLC/PRF/5 cells showed a significantly higher percentage of annexin V-positive cells than did control cells. Consistent with this result, the well-known apoptosis protein marker, cleaved Caspase 3, were markedly increased by lncPARP1 knockdown (Figure 2E).

Knockdown of lncPARP1 represses migration and invasion in HCC cells

We next investigated the role of lncPARP1 in HCC cell migration and invasion. Cell motility was measured by migration assay. The results showed that depletion of lncPARP1 suppressed the migratory capacity of both Bel-7402 and PLC/PRF/5 cells (Figure 3). We then used Matrigel-coated Transwell experiments to examine HCC cell invasion, and the result revealed that the number of Bel-7402 and PLC/PRF/5 cells invading through membrane was significantly reduced after lncPARP1 silencing (Figure 3).

lncPARP1 positively modulate PARP1 expression

lncRNAs act in cis or trans to regulate the expression of neighboring protein-coding genes [19]. LncPARP1 is an intergenic lncRNA, which was localized downstream of PARP1 gene. Therefore, we speculated that lncPARP1 could affect PARP1 expression. To test this hypothesis, we detected the mRNA and protein levels of PARP1 in Bel-7420 and PLC/PRF/5 expressing lncPARP1 shRNAs. Interestingly, depletion of lncPARP1 significantly repressed both mRNA and protein levels of PARP1 (Figure 4A,B). We further clarified the pathological relationship between lncPARP1 and PARP1 expression. We examined PARP1 mRNA expression in 70 pairs of HCC and paratumor tissues by qRT-PCR. The results showed that PARP1 mRNA expression was much higher in HCC tissues than that in adjacent non-tumor
Figure 5. Effects of lncPARP1 knockdown on HCC growth in vivo

(A) Upper, representative images of tumors formed in nude mice subcutaneously injected with control and lncPARP1-knockdown PLC/PRF/5 cells; Below, tumor weight measured 6 weeks after injecting. (B) Proliferation marker PCNA expression in tumor tissues formed by control and lncPARP1-knockdown PLC/PRF/5 cells was examined by IHC. (C) lncPARP1 and PARP1 RNA expression of in tumor tissues formed by control and lncPARP1-knockdown PLC/PRF/5 cells was analyzed by qRT-PCR. (D) The PARP1 protein expression in tumor tissues formed by control and lncPARP1-knockdown PLC/PRF/5 cells was examined by performing IHC staining. *P<0.05.

Deletion of lncPARP1 suppresses HCC growth in vivo

To further confirm the function of lncPARP1 during process of HCC growth, we used a xenograft mouse model that was generated by subcutaneously injecting control and lncPARP1-knockdown PLC/PRF/5 cells into nude mice. Xenografted tumors derived from lncPARP1-knockdown PLC/PRF/5 cells had smaller volumes and expressed lower level of PCNA than tumors derived from control cells (Figure 5A,B). Consistent with in vitro observation, both mRNA and protein levels of PARP1 in lncPARP1 knockdown tumors were much lower than that in control tumors (Figure 5C,D). Collectively, our findings suggest that lncPARP1 promotes tumor growth through up-regulation of PARP1 in vivo.
**Discussion**

LncRNAs have emerged as critical regulators in HCC tumorigenesis and progression. Nevertheless, the biological functions and underlying mechanistic details for most LncRNAs in HCC is still elusive. The key finding of our current study is that a novel LncRNA lncPARP1 is significantly up-regulated in HCC tissues. Increase in lncPARP1 expression is closely associated with age, AFP level, tumor size, and recurrence of HCC patients. High-level lncPARP1 expression is a potential predictor for poor tumor-free and overall survival of HCC patients. We then determined the relationship between lncPARP1 expression and malignant phenotypes of HCC cells using loss-of-function approaches. Knockdown of lncPARP1 inhibited proliferation, migration, and invasion, while induced apoptosis in HCC cells. This is the first time to report the clinical and functional significance of lncPARP1 expression contributing to HCC tumorigenesis and progression.

PARP1 is the major member of Parps family to promote PAR chain formation through DNA-dependent modulation. PARP1-mediated poly(ADP-ribosylation) (PARylation) is a post-translational modification, which regulates protein–protein interaction and transcription [20,21]. Abnormally expressed PARP1 is involved in carcinogenesis. Specifically, PARP1 is crucial in eliciting the protection against genotoxic effects via regulating the DNA repair mechanism, PARP1 interacts with P53 and promotes its PARylation to inhibit binding affinity of p53 to target genes during apoptosis program [22], implying that lncPARP1 may regulate the DNA repair in response to DNA damage through PARP1–p53 signaling. PARP1 also plays important role in mesenchymal–epithelial transition (EMT) which promotes tumor metastasis. PARP1 associates with EMT-related transcription factor Snail. PARP1 mediated PARylation affects the stability of Snail to modulate EMT process [23]. Thus, these studies indicate a crucial role of PARP1 in cancers development. Recently, it has been reported that some LncRNAs regulate cancer progression through interaction with PARP1. For example, overexpression of LncRNA FOXD3-AS1 induces neuronal differentiation and decreases the aggressiveness of neuroblastoma cells through interacting with PARP1 and inhibiting the activation of CCCTC-binding factor (CTCF) [24]. However, to the best of our knowledge, whether LncRNAs take part in the increase in PARP1 expression in cancer remains unknown. Our present study showed that knockdown of lncPARP1 significantly suppressed both mRNA and protein levels of PARP1 expression in vitro and in vivo. Moreover, a positive correlation between lncPARP1 and PARP1 expression in HCC tissues was observed, suggesting that PARP1 was a bona fide target gene of lncPARP1. Unfortunately, we did not reveal the exact mechanism of PARP1 expression induced by lncPARP1. Previous studies demonstrated that intergenic LncRNAs exert functions through interaction with epigenetic modifier to regulate nearby protein-coding gene expression [25]. For example, LncRNA-HEIH was associated with EZH2 and repressed EZH2 target genes, such as p16, p21, and p57 [26]. LncTCF7 activated TCF7 expression to promote self-renewal of HCC stem cells through interaction with the SWI/SNF complex [25]. Therefore, we suspect that lncPARP1 modulates PARP1 expression in such a manner, which needs further investigation.

**Conclusion**

In conclusion, we characterized a new functional LncRNA lncPARP1 that regulates the expression of PARP1 and is a novel molecule involved in the progression of HCC. Our findings suggest that lncPARP1 might be used as a promising prognostic and therapeutic marker of HCC.

**Acknowledgements**

We thank Prof Jian Zhou (Zhongshan Hospital of Fudan University) for providing MHCC-97h cells.

**Funding**

This work was supported by the National Natural Science Foundation of China [grant numbers 81672418, 81702351, 81702348, 81572335]; the Research Project of Health and Family Planning [grant numbers 2018-2-64, 2018-ZQN-84]; the Natural Science Foundation of Fujian [grant numbers 2017-2-101, 2018J01389, 2018J01398, 2014D022]; and the Science and Technology Project of Xiamen [grant numbers 3502Z20164023, 3502Z20164022].

**Competing interests**

The authors declare that there are no competing interests associated with the manuscript.

**Author contribution**

All the authors performed the experiments. Z.Y. and C.X. participated in the design of the study. H.Q. and Y.L. participated in data analysis and statistical analysis. C.X. and Y.L. wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations
ACTB, beta-actin; AFP, α-fetoprotein; CCK-8, cell counting kit-8; DMEM, Dulbecco's Modified Eagle's medium; EMT, mesenchymal–epithelial transition; EZH2, Enhancer of zeste homolog 2; HCC, hepatocellular carcinoma; HRP, Horseradish peroxidase; lincRNA, long intergenic noncoding RNA; lncRNA, long noncoding RNA; PAR, poly (ADP-ribose); PARP1, PAR poly-merase 1; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative real-time PCR; RIPA, Radio Immunoprecipitation Assay; SWI/SNF, Switch/Non-Fermentable; UFC1, ubiquitin-fold modifier conjugating enzyme 1.

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