Original Article
Up-regulation of CLIC1 activates MYC signaling and forms a positive feedback regulatory loop with MYC in Hepatocellular carcinoma

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Received June 30, 2020; Accepted July 5, 2020; Epub August 1, 2020; Published August 15, 2020

Abstract: Hepatocellular carcinoma (HCC) is leading cause of tumor-related deaths worldwide. The intracellular chloride channel protein (CLIC1) plays a role in the occurrence and progression of HCC, although the underlying mechanisms are still unclear. We evaluated the CLIC1 mRNA and protein levels in both patient tissues and HCC cell lines, and analyzed the correlation between CLIC1 expression and clinical features. The biological function of CLIC1 in HCC was examined in vivo and in vitro. The upstream regulatory factors were identified by bioinformatics programs, and downstream mechanisms affecting HCC behavior have also been explored and validated. CLIC1 was up-regulated in HCC tissues and cell lines, and promoted the proliferation, invasion and migration of HCC cells in vivo and in vitro. TP53 was identified as the upstream transcription factor of CLIC1. MiR-122-5p also regulated CLIC1 levels by degrading the transcripts. More importantly, we found that the increased CLIC1 was significantly related to the activation of MYC signaling. By binding with MYC, CLIC1 enhanced the transcription activity of MYC to downstream genes, rather than by altering its expression. Finally, a positive feedback regulatory loop between CLIC1 and MYC was established.

Keywords: Hepatocellular carcinoma, CLIC1, MYC, TP53, miR-122-5p

Introduction
Liver cancer is responsible for a substantial proportion of cancer-related deaths worldwide [1, 2], and ranks second in China in terms of incidence [3]. Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer, accounting for 70-90% of all cases [4], and associated with high recurrence and metastasis rates. Risk factors of HCC include age, sex and cirrhosis, although the primary causes are chronic hepatitis due to virus infection and alcohol [5]. Due to the absence of specific symptoms, most patients are diagnosed at the advanced stage that precludes surgical resection. Therefore, the molecular mechanisms underlying HCC progression and metastasis need to be elucidated in order to develop new prevention and treatment strategies.

Chloride channels regulate membrane potential, ion homeostasis, transmembrane transport and cell volume [6], and are thus crucial for tumor invasion and metastasis [7, 8]. The chloride intracellular channel 1 (CLIC1) [9] is upregulated in prostatic adenocarcinoma [10], gastric carcinoma [11] and colorectal carcinoma [12], and an independent prognostic biomarker for gallbladder [13] and colorectal cancer [14]. Furthermore, CLIC1 promotes HCC migration and invasion by targeting maspin [15], although the molecular mechanism is unclear.

We found that CLIC1 was significantly upregulated in HCC tissues and correlated with poor prognosis, and promoted HCC cell proliferation and metastasis via the activation of MYC signaling and a positive feedback regulatory loop with MYC was confirmed.
Materials and methods

Tissue samples and cell culture

Eighty paired HCC and non-tumor liver tissues were harvested from patients at the Zhongnan Hospital, Wuhan University (Hubei province, China) with informed consent. The study protocol was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Hospital’s Protection of Human Subjects Committee. All cell lines were procured from the cell bank of the Chinese Academy of Science (Shanghai, China), and cultured in DMEM/HIGH GLUCOSE (GE, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C under 5% CO2. Cells in the logarithmic phase were used for the experiments.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from the tissues and cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The Takara Reverse Transcriptase cDNA Synthesis Kit (Takara, Tokyo, Japan) was used to synthesize first-strand cDNA, and Takara SYBR Green PCR Kit ((Takara, Tokyo, Japan) for qRT-PCR. Each 20 µL reaction mix contained 500 ng cDNA and the specific reverse and forward primers. All primer sequences are listed in the Additional file 5: Table S1.

Chromatin immunoprecipitation (Chip) assay

Magna ChiP-q™ Chromatin Immunoprecipitation Kit (Millipore, Billerica, USA) was used for detecting protein-chromatin interactions. Briefly, the cells were fixed with formaldehyde and sonicated, and incubated with the target protein. The cross-linked DNA fragments were then released from the co-precipitated complexes, purified, and amplified by PCR.

Dual luciferase reporter activity assay

Dual luciferase assay was performed as per standard protocols (Promega, Madison, USA). The luciferase gene was cloned downstream of the wild-type or mutant 3’UTR region of the target gene, and co-transfected into HCC cells with the siRNA or miRNA mimics. After 48 hours, the cells were lysed and the firefly luciferase activity was measured using a microplate reader with Renilla luciferase as the internal reference.

Immunoprecipitation

The IP Kit Dynabeads Protein G Per Maker protocol (ThermoScience, CAT # 10007D) was used for immunoprecipitation assay. Briefly, suitably treated cells were harvested and lysed on ice using the IP lysis buffer (containing protease inhibitors) for 30 min. The lysates were precipitated at 12000 g for 30 min, and the protein content in the supernatant was measured. Equal amount (1 µg) of proteins per sample was incubated overnight with 10-50 µl protein A/G-beads at 4°C. The immuno-complex was precipitated at 3000 g for 5 min at 4°C, and the supernatant was washed 3 to 4 times with 1 ml lysis buffer. Finally, 15 µl of each sample was boiled in 2× SDS loading buffer for 10 minutes, and analyzed by Western blotting as per standard protocols. Antibodies used in this study are listed in the Additional file 6: Table S2.

Establishment of orthotopic HCC and lung metastasis models

All animal experiments were approved by the animal ethics committee of Wuhan University’s Institutional Animal Care and Use Committee of Center, and performed in compliance with institutional protocols. Male BALB/c nude mice (aged 4-6 weeks) were anesthetized and injected intra-hepatically with 1×10⁶ HCC cells by laparotomy to induce orthotopic tumors. The mice were sacrificed 4 weeks later for routine analysis. Lung metastasis model was established via intravenous injection of 3×10⁶ HCC, and the lungs were harvested 4 weeks later for immunohistochemistry.

Bioinformatics analysis

The HCC transcriptomic and clinical data were downloaded from the GEO database, tumor genome map (TCGA), liver cancer data set (LIHC) and GEPIA (http://gepia.cancer-pku.cn). The TargetSAN (http://www.targetscan.org/), miRDB (http://mirdb.org/miRDB/), miRNA (Http://www.microrna.org/microrna/home.do/) and Genecardhttps (https://www.genecards.org/) programs were used to predict the transcription factors and miRNAs involved in the regulation of CLIC1.

Statistical analysis

All quantitative data were presented as the mean ± SD of at least three independent experiments. Two-sided Student’s t-test and one-way
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ANOVA (SPSS statistical package, version 12; SPSS Inc.) were used to respectively compare two and multiple groups. Kaplan-Meier method and Cox’s proportional hazards regression model were used to evaluate overall survival. P<0.05 was considered statistically significant.

Results

CLIC1 is upregulated in HCC and predicts worse survival

We screened the GSE47656 dataset from GEO, which contains expression data from 5 normal livers, HCC and PVTT samples each, to identify HCC-specific protein markers. As shown in the heat map in Figure 1A, CLIC1 was significantly upregulated in HCC and PVTT relative to normal liver. Similar analysis of the GSE40396 dataset further revealed that CLIC1 levels were higher in the metastatic versus non-metastatic HCC tissues (Figure 1B), indicating that CLIC1 likely plays a role in HCC occurrence and metastasis. Besides, CLIC1 was correlated to the histologic grade of HCC (Figure 1C, Additional file 1: Figure S1). Consistent with the in-silico data, CLIC1 mRNA (Figure 1D) and protein (Figure 1E, 1F) levels were significantly higher in 80 freshly-resected HCC tissues compared to the corresponding para-tumor tissues, as well as in HCC cell lines.
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The biological function of CLIC1 was determined by gene knockdown in the Huh7 and SK-Hep1 cells, and overexpression in HCCLM3 cells. Stably transfected cell lines were verified by RT-PCR and Western blotting (Figure 2A). Knocking down CLIC1 significantly inhibited the proliferation of HCC cells (Figure 2B, 2C), and impaired invasion and wound migration in vitro (Figure 2D, 2E). Consistent with this, epithelial mesenchymal transition (EMT) was inhibited in the CLIC1-knockdown cells (Figure 3A). In contrast, CLIC1 overexpression significantly increased the proliferation, invasion and migration of HCCLM3 cells (Additional file 2: Figure S2).

CLIC1 knockdown repressed tumor growth and metastasis in vivo

To further explore the role of CLIC1 in HCC tumorigenesis, an orthotopic HCC mouse model was constructed using control and CLIC1-knockdown Hep3B cells (Figure 3B). Compared to the control, tumors lacking CLIC1 imparted weaker PET-CT signals and were remarkably smaller in terms of volume (Figure 3C-E). Consistent with this, the CLIC1-knockdown tumors showed lower Ki67 expression corresponding to a lower proliferative index (Figure 3F). Furthermore, CLIC1 knockdown also decreased the number of pulmonary tumor nodules in the lung metastasis model compared to the control group (Figure 3G, 3H).

TP53 depletion suppressed CLIC1 in HCC

Given that aberrant transcription events [16] can activate multiple oncogenic signaling pathways, we next screened for the putative upstream transcription factors of CLIC1 using PROMO and Genecard, and identified TP53 and SP1 (Figure 4A). RNA-seq data of HCC samples indicated a significant positive correlation between SP1 and CLIC1 (R=0.42; Figure 4B, 4C). In addition, both wild type and mutant P53 were positively correlated with CLIC1 (>0.42), and mutant P53 showed the strongest correlation (R=0.62; Figure 4D). Furthermore, TP53 was significantly upregulated in HCC (Figure 4F), and the mutant P53 showed greater correlation with CLIC1 expression levels compared to wild-type P53 (Figure 4E). Consistent with this, knocking down TP53 in Huh7 and SK-Hep1 cells significantly downregulated CLIC1 mRNA and protein levels (Figure 4G). The binding of TP53 to the promoter region of CLIC1 gene was also verified by

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of cases</th>
<th>CLIC1 expression</th>
<th>P value</th>
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<tr>
<td></td>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
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<td></td>
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<td>20 (0.25)</td>
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<td>&lt;65</td>
<td>37 (0.462)</td>
<td>18</td>
<td>19</td>
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<tr>
<td>≥65</td>
<td>43 (0.538)</td>
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</tr>
<tr>
<td>&lt;5</td>
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<td>33</td>
</tr>
<tr>
<td>≥5</td>
<td>19 (0.238)</td>
<td>12</td>
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<tr>
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<td>9</td>
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<tr>
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<td>16 (0.2)</td>
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<td>61 (0.762)</td>
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<td>TNM stage</td>
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<tr>
<td>I+II</td>
<td>61 (0.762)</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>III+IV</td>
<td>19 (0.238)</td>
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<td>BCLC stage</td>
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<td>15 (0.188)</td>
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<td></td>
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<td>66 (0.825)</td>
<td>29</td>
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<td>14 (0.175)</td>
<td>11</td>
<td>3</td>
</tr>
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</table>

BCLC, Barcelona Clinic Liver Cancer; PVTT, portal vein tumor thrombus. Bold italics indicate statistically significant values (*P<0.05).
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Table 2. Univariate and multivariate analyses of clinicopathological characteristics, and CLIC1 with overall survival

<table>
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<th>Characteristics</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<td>Hazard Ratio</td>
<td>95% CI</td>
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<tr>
<td>Gender</td>
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<td>0.475-2.29</td>
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<td>Age</td>
<td>1.019</td>
<td>0.529-1.961</td>
</tr>
<tr>
<td>Tumor Size (cm)</td>
<td>1.330</td>
<td>0.654-2.705</td>
</tr>
<tr>
<td>HBV infection</td>
<td>1.170</td>
<td>0.55-2.489</td>
</tr>
<tr>
<td>Multiple nodules</td>
<td>1.210</td>
<td>0.569-2.575</td>
</tr>
<tr>
<td>AFP (μg/L)</td>
<td>1.061</td>
<td>0.542-2.075</td>
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<tr>
<td>Cirrhosis</td>
<td>2.085</td>
<td>0.81-5.365</td>
</tr>
<tr>
<td>TNM stage</td>
<td>3.555</td>
<td>2.104-6.007</td>
</tr>
<tr>
<td>BCLC stage</td>
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<td>2.431-7.923</td>
</tr>
<tr>
<td>PVTT</td>
<td>4.738</td>
<td>2.243-10.012</td>
</tr>
<tr>
<td>CLIC1</td>
<td>1.389</td>
<td>1.144-1.685</td>
</tr>
</tbody>
</table>

HR, Hazard ratio; CI, Confidence interval. Bold italics indicate statistically significant values (*P<0.05).

miR-122-5p inhibition upregulated CLIC1 in HCC

miR-122-5p and miR-3818 were identified as the upstream regulators of CLIC1 (Figure 5A), of which only the former was negatively correlated with CLIC1 (R=-0.44, P<0.001; Figure 5B). The unpaired HCC data from TCGA and the paired data of GSE108724 both indicated that miR-122-5p is downregulated in HCC, and correlates with worse survival (Figure 5C, 5E). The prognostic role of miR-122-5p was also confirmed in the clinical HCC samples (Figure 5D). Furthermore, the miR-122-5p mimics significantly downregulated CLIC1 in HCC cells, while the inhibitor had the opposite effect (Figure 5F, 5G). The interaction between miR-122-5p and CLIC1 was verified by luciferase reporter assay, and the fluorescent activity reduced by a mutant CLIC1 binding site was restored upon transfecting miR-122-5p mimics (Figure 5H). Finally, miR-122-5p also counteracted CLIC1-induced proliferation, invasion and migration of HCC cells (Additional file 3: Figure S3). Taken together, miR-122-5p directly targets and downregulates CLIC1 in HCC cells.

**CLIC1 modulates MYC-related genes and forms a positive feedback loop with MYC**

Single gene GSEA enrichment analysis showed that CLIC1 overexpression was closely related to MYC targets_1, PI3K-AKT-mTOR signal pathway, DNA repair etc. (Figure 6A). We intersected the gene set of MYC targets_1 with the MYC ENCODE Chip-seq data, and identified 12 genes that are likely regulated by CLIC1 (Figure 6B), of which EIF3D, HNRNPA1, EIF3B, RPS3 and YWHAE showed the highest correlation coefficient with CLIC1 (Figure 6C). CLIC1 knockdown did not significantly affect MYC (data not shown) but downregulated all five MYC target genes (Figure 6D), and also decreased their binding to MYC (Figure 6E). In addition, anti-CLIC1 co-IP showed direct binding of CLIC1 and MYC (Figure 6F), in order to further confirm the regulatory relationship among CLIC1, MYC and 5 MYC target genes, we also conducted rescue experiments. (Additional file 4: Figure S4).

Although overexpression of CLIC1 in HCC cell lines did not affect MYC levels (data not shown), knocking down or overexpressing MYC altered the expression of CLIC1 accordingly (Figures 6C, 7A). Luciferase reporter assay further revealed a MYC binding site in the promoter region of CLIC1, and a mutation at this site reversed the decrease in fluorescence activity caused by MYC-siRNA transfection (Figure 7B). MYC Chip-PCR directly showed the binding of MYC to the promoter region of CLIC1 (Figure 7C). Taken together, MYC not only upregulates CLIC1 but also other downstream genes by binding to CLIC1, indicating a positive feedback between MYC and CLIC1 (Figure 7D).

**Discussion**

HCC is the main cause of cancer-related mortality worldwide [17, 18], and is primarily caused
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(A) Relative CLIC1 expression in Huh7 and SK-Hep1 cells. si-NC, siCLIC1 #1, siCLIC1 #2, and siCLIC1 #3 were used for knockdown experiments. Vector and CLIC1 were used as controls.

(B) OD 450 values over time for Huh7 and SK-Hep1 cells. si-NC, siCLIC1 #1, and siCLIC1 #2 were used for knockdown experiments.

(C) Number of colonies in Huh7 and SK-Hep1 cells. si-NC, siCLIC1 #1, and siCLIC1 #2 were used for knockdown experiments.

(D) Cell numbers in Huh7 and SK-Hep1 cells. si-NC, siCLIC1 #1, and siCLIC1 #2 were used for knockdown experiments.

(E) Wound healing percentage (% normalized to si-NC) in Huh7 and SK-Hep1 cells. si-NC, siCLIC1 #1, and siCLIC1 #2 were used for knockdown experiments.
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Figure 2. CLIC1 knockdown inhibits the proliferation, invasion and migration of HCC. A. CLIC1 knockdown and overexpression in Huh7, SK-Hep1 and HCCLM3 cell lines. B. C. CCK8 and colony formation assays with the suitably transfected HCC cells. D, E. Invasion and migration of the suitably transfected HCC cells. *P<0.05, **P<0.01, ***P<0.001.
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**Figure 3.** CLIC1 knockdown repressed tumor growth and metastasis in vivo. A. Expression levels of EMT markers (E-cadherin, claudin-1, N-cadherin, vimentin, and fibronectin) and MMPs (MMP-2, MMP-9) in HCC cells after CLIC1 knockdown and overexpression. B. CLIC1 knockdown efficiency in Hep3B cells. C, D. Representative PET/CT images of orthotopic HCC tumors in nude mice. E. Tumor volume in the indicated groups. F. Representative images of HE-stained and Ki67 immuno-stained tumor tissues in the indicated groups. G, H. Lung specimens and HE stained images of the lung metastasis model showing the number of lung tumor nodules. **P<0.01.
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A

PROMO

TP53

SP1

GeneCard

27
4

B

TCGA LIHC

R = 0.42, p < 2.2e-16

TP53 expression (log2(count+1))

CLIC1 expression (log2(count+1))

C

TCGA LIHC

R = 0.36, p = 2.6e-12

TP53 expression (log2(count+1))

CLIC1 expression (log2(count+1))

D

Wild type mutation

Missense mutation

E

TCGA LIHC

***

TP53 expression (log2(count+1))

CLIC1 expression (log2(count+1))

F

TCGA LIHC

Tumor Non-tumor

G

si-NC

siTP53 #1

siTP53 #2

si-NC

siTP53 #1

siTP53 #2

TP53

CLIC1

GAPDH

Huh7

SK-Hep1

53KDa

32KDa

36KDa

G

H

CLIC1 promoter

Luc

WT 5’ -AACATGGCTGGGCGTGGT-3’

MUT 5’ -AACATGGAGTTTCGTGGT-3’

I

**

Primer #1

% input

Huh7

HepG2

**

Primer #2

% input

Huh7

HepG2

***

IgG

IP

Control

siTP53 #1

siTP53 #2
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by chronic hepatitis B virus infection in China [19]. Surgery is routinely performed on HCC patients but the high postoperative recurrence and metastasis rates severely reduce the quality of life and shorten the five-year survival rate. Therefore, it is vital to identify novel therapeutic targets and diagnostic markers in order to improve prognosis.

We found that CLIC1 was highly expressed in HCC tissues, especially in the metastatic cases, and correlated with worse survival. In addition, CLIC1 was also closely related to the tumor stage and the occurrence of PVTT, indicating that CLIC1 functions as an oncogene in HCC. Furthermore, TP53 was identified as the transcription factor of CLIC1, and the expression levels of the latter were higher in mutant p53 compared to wild-type P53 HCC tissues. Since CLIC1 was precipitated by the anti-TP53 antibody in both P53 mutant (Huh7) and wild type (HepG2) HCC cells, we could not conclude whether either can bind to the promoter region of CLIC1 with greater efficiency. Whereas wild-type p53 has a short half-life in situ [20], the mutant protein can form stable complexes with viral proteins [21], the wild type counterpart [22] and heat shock proteins [23] and accumulate over a period of time. The mechanistic basis of the higher expression of CLIC1 in P53-mutant versus wild type HCC needs further investigation. DNA methylation is an important epigenetic regulatory mechanism, and we observed hypomethylated CLIC1 promoter in the HCC samples from TCGA database compared to normal liver samples. There are four putative methylation sites in the promoter region of CLIC1 (Additional file 1: Figure S1), which we also confirmed by methylation-specific PCR. However, the molecular mechanisms need to be further explored.

Interestingly, several MYC target genes were strongly correlated with CLIC1 in the HCC tissues. MYC is a proto-cancer transcription factor that participates in cell growth, proliferation, apoptosis and metabolism, and cooperates with other oncogenic pathways to trigger malignant transformation [24]. The MYC signaling pathway is activated during the progression of HCC [25-27], although that is not related with MYC overexpression or amplification [25, 28]. Our findings suggest that the activated MYC signaling was induced by the upregulation of CLIC1 rather than MYC protein itself, which likely plays an important role in the tumorigenesis of HCC [29]. P53 is a multifunctional protein and participates in various life activities. The function of P53 is to act as a checkpoint to respond to endogenous and exogenous pressure signals, and integrate upstream signals as the central node of the signal transduction network [30], responsible for reducing the feasibility of tumorigenesis and other pathologies. This explained why mutant P53 is common in human tumors. P53 mutations can be observed in about 50% of tumors, and families that inherit P53 mutations have a 100% probability of developing cancer [31]. p53 monitors genomic integrity at the cell cycle checkpoint. Cells lacking p53 may undergo chromosomal amplification and neoplastic polyploidy. Studies have found that C-myc overexpression and p53 deletion synergistically promote genomic instability [30], and the restoration of P53 function in cells that overexpress c-myc will lead to apoptosis. P53 and MYC form a complex regulatory system functionally, the normal function of P53 is a key factor to maintain the balance of this system. Overexpression of MYC promotes cell immortality, reduces the demand for growth factors, and weakens the stability of genome, leading to tumorigenesis. It has also been reported that MYC, as an independent cell cycle regulator, involved in the occurrence and development of tumors [32].

The oncogenic role of CLIC1 in other tumors has gained considerable attention in recent years. For instance, the CLIC1 blocker IAA94 inhibited the invasion and migration of the human colorectal cancer cell line LOVO by enhancing the production of intracellular ROS [33]. In addition, metformin can suppress the
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Figure 5. Inhibition of miR-122-5p upregulates CLIC1 in HCC. A. MiR-122-5p and miR-3818 putatively bind to the 3'UTR region of CLIC1 mRNA. B. Correlation between miR-122-5p and CLIC1. C. MiR-122-5p levels in HCC samples in TCGA LIHC datasets. D. Correlation between CLIC1 and miR-122-5p in clinical HCC samples. E. Correlation between miR-122-5p expression and survival. F, G. CLIC1 expression levels in cells transfected with miR-122-5p mimics or inhibitor. H. Luciferase reporter assay showing fluorescent activity in cells co-transfected with miR-122-5p mimics and wild-type or mutant CLIC1 promoter. *P<0.05; **P<0.01; ***P<0.001.
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A

TCGA LIHC

B

HepG2

K562

C

AS-30

MC-7

MYC Chip-seq

CNRP+EIF3B,YWAHE,RP33

TRIM32,PWP1,EIF3D,SET

GSEA MYC targets

CNRP+MRPL23,HNRNA1,ETF1

D

EIF3B

RPS3

HNRNA1

YWAHE

HBB

E

F

input

IP

MYC

IgG

input

IP

MYC

IgG

CLIC1

input

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MYC

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CLIC1
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Figure 6. CLIC1 modulates MYC-related genes. A. Enriched pathways associated with CLIC1 in single-gene GSEA. B. 12 MYC-related genes regulated by CLIC1. C. Pearson correlation analysis of EIF3B, EIF3D, HNRNPA1, RPS3, YWHAE and MYC with CLIC1. D. EIF3B, EIF3D, HNRNPA1, RPS3, YWHAE and HBB expression in CLIC1-knockdown cells. E. Binding of MYC to EIF3B, EIF3D, HNRNPA1, RPS3 and YWHAE in CLIC1-knockdown cells. F. Co-IP assay showing binding of CLIC1 and MYC. *P<0.05; **P<0.01; ***P<0.001.

Figure 7. MYC promotes the transcription of CLIC1. A. CLIC1 expression levels in MYC-knockdown or overexpressing HCC cells. B, C. Dual fluorescein reporter and Chip-PCR assay showing MYC binding to the CLIC1 promoter region. D. Schematic diagram showing the positive feedback loop between CLIC1 and MYC, and the regulatory action of p53 and miR-122-5p. *P<0.05; **P<0.01.

The growth of glioblastoma stem cells by blocking the channel function of CLIC1 [34]. Consistent with the above, our findings also support CLIC1 as a promising therapeutic target in HCC.

In conclusion, CLIC1 is overexpressed in HCC and predicts worse outcome. It is regulated by TP53 and miR-122-5p, and likely promotes HCC through a positive feedback loop with MYC.

Acknowledgements

We thank the teachers from the Scientific Research Center of Zhongnan hospital of Wuhan University for their help in this study. Our work was supported by Scientific Research Project of Hubei Province Health commission (WJ20-17Z007).

Disclosure of conflict of interest

None.

Abbreviations

HCC, Hepatocellular carcinoma; CLIC1, chloride intracellular channel 1; RT-qPCR, Real-time quantitative polymerase chain reaction; TCGA, The Cancer Genome Atlas; GEO, Gene Expre-
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Siom Omnibus; GSEA, Gene set enrichment analysis; TP53, Tumor protein p53; MYC, v-myc avian myelocytomatosis viral oncogene homolog; HE, Hematoxylin-eosin; IHC, Immunohis-tochemistry; LIHC, Liver Hepatocellular Carcinoma; SDS-PAGE, Sodium dodecyl sulfate-poly-acrylamide; PVDF, Polyvinylidene difluoride; sh-RNA-CLIC1, Small hairpin RNA expression vector targeting human CLIC1 gene; sh-RNA-NC, Scrambled shRNA; siRNA, small interfering RNAs.

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References

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Table S1. Primers and oligonucleotides sequences

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<th>siRNAs</th>
<th>Interfering oligonucleotides</th>
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**PCR primers**

| PCR-CLIC1 #1-F | ATATGCTAGACAGTGAGGTGAG                                  |
| PCR-CLIC1 #1-R | CTGGGACTACAGGTGAG                                       |
| PCR-CLIC1 #2-F | ATGCTAGACAGTGAGGAG                                     |
| PCR-CLIC1 #2-R | GCTGGGACTACAGGTTG                                      |
| PCR-MYC-F      | CGCTTCTCTGAAAGGCTCTCTTTG                               |
| PCR-MYC-R      | GATCGTGACGTCAAGGTCATAAGTT                              |
| PCR-TP53 -F    | CCCCCTCAGACTCTTATCC                                    |
| PCR-TP53 -R    | TGCTACAGTCAGGAGCCAACT                                  |
| PCR-EIF3B -F   | ATCTGGGACATCTTTACGG                                   |
| PCR-EIF3B -R   | TGCTAAGCGTATCCAGGG                                    |
| PCR-EIF3D -F   | TGGACAGGCTCACGGTATCC                                   |
| PCR-EIF3D -R   | GTGGTTGATGAGTGTCATGG                                  |
| PCR-HNRNPA1 -F | ACCTTTGAGACCAGTGACTCC                                  |
| PCR-HNRNPA1 -R | CCAGAACCATCTTCAGGACAC                                 |
| PCR-RPS3 -F    | GTGGTGCTCAGACAGGGAATGG                                |
| PCR-RPS3 -R    | GGCGCATATCTCATCTTGG                                   |
| PCR-YWHAE -F   | CCACAGGGAACGAGGAGA                                    |
| PCR-YWHAE -R   | GCACCGGTCAAGGGAATTAAG                                  |
| PCR-HBB -F     | GGAATGATGTTTCTCCGAG                                   |
| PCR-HBB -R     | TTGAGGTGTCCAGGAG                                       |
| PCR-GAPDH-F    | AACCTTCAGAATTTCGCT                                       |
| PCR-GAPDH-R    | GCTGTCACTTCAGGCTTCC                                    |
| PCR-miR-122-5p-F | GGGTGAGTTGTAGAATGG                                    |
| PCR-miR-122-5p-R | CAGTGGCGTCTCAGGAG                                     |
**Table S2. Antibodies used in this study**

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<tr>
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<tr>
<td>HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L)</td>
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</table>
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A

Expression of CLIC1 across TCGA samples

B

F value = 2.56
P(>F) = 0.0138

C

Expression of CLIC1 in LIHC based on nodal metastasis status

D

Promoter methylation level of CLIC1 in LIHC

E

Overall Survival
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Figure S1. A. The mRNA level of CLIC1 in 24 kinds of tumor types from TCGA. B. Differential expression of CLIC1 in tumor grades. C. Expression of CLIC1 based on nodal metastasis status. D. Downregulation of the methylation level of CLIC1 promoter region in HCC samples, and the predicted methylation site (CpG island). E. Kaplan-Meier analysis of overall survival in 182 HCC patients from the TCGA dataset.

Figure S2. A, B. CCK 8 and colony formation assay in HCCLM3 cells with or without CLIC1 overexpression. C. The result of EdU cell proliferation assay. D. Effect of overexpressing CLIC1 on the invasion and migration of HCCLM3 cells. E, F. Representative images of tumors removed from nude mice implanted with CLIC1 overexpressed HCCLM3 cells (n=6) or control cells (n=6), and the tumor growth curve. *P<0.05, **P<0.01.
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Figure S3. A. CLIC1 mRNA and protein levels after transfection of miR-122-5p. B, C. CCK 8 and EdU cell proliferation assay in HCCLM3-CLIC1 cells with or without miR-122-5p transfected. D. The effect of miR-122-5p transfection on cell invasion and migration ability. *P<0.05, **P<0.01.
Figure S4. A. Relative mRNA expression of EIF3B, EIF3D, HNRNPA1, RPS3, YWHAE and HBB, in HCCLM3 with or without CLIC1 overexpressed. B. CLIC1 enhanced the transcription ability of MYC to downstream genes. C. The decrease of downstream gene transcription induced by MYC knockdown could be partially compensated by transfection of CLIC1 plasmid. D. Interference with CLIC1 could partially counteract the enhanced transcription of MYC downstream gene caused by MYC plasmid transfection. *P<0.05, **P<0.01, ***P<0.001.