PIK3CA mutations-mediated downregulation of circLHFPL2 inhibits colorectal cancer progression via upregulating PTEN

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Abstract

Background: PIK3CA mutation and PTEN suppression lead to tumorigenesis and drug resistance in colorectal cancer (CRC). There is no research on the role of circular RNAs (circRNAs) in regulating PIK3CA mutation and MEK inhibitor resistance in CRC.

Methods: The expression of circLHFPL2 in PIK3CA-mutant and wild-type cells and tissues was quantified by RNA-sequencing and qRT-PCR. CCK-8 assay and colony formation assay were used to evaluate cell viability. Annexin V/PI staining was implemented to assess cell apoptosis. Luciferase assay, biotin-coupled microRNA capture, and RIP assay were used to validate the interaction among potential targets. Western blotting and qRT-PCR assays were used to evaluate the expression of involved targets. Xenograft tumor in a nude mouse model was used to explore the role of circRNAs in vivo.

Results: RNA sequencing defined downregulated expression of circLHFPL2 in both PIK3CAH1047R (HCT116) and PIK3CAE545K (DLD1) cells. CircLHFPL2 was also downregulated in PIK3CA-mutant CRC primary cells and tissues, which was correlated with poor prognosis. CircLHFPL2 was mainly localized in the cytoplasm and its downregulation was attributed to the PI3K/AKT signaling pathway activated by phosphorylating Foxo3a. CircLHFPL2 inhibited PI3KCA-Mut CRC progression both in vitro and in vivo. Furthermore, our work indicated that circLHFPL2 acts as a ceRNA to sponge miR-556-5p and miR-1322 in CRC cells and in turn modulate the expression of PTEN. Importantly, circLHFPL2 was able to overcome PIK3CA-mediated MEK inhibitor resistance in CRC cells.

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Conclusions: Downregulation of circLHFPL2 sustains the activation of the PI3K/AKT signaling pathway via a positive feedback loop in PIK3CA-mutant CRC. In addition, downregulation of circLHFPL2 leads to MEK inhibitor resistance in CRC. Therefore, targeting circLHFPL2 could be an effective approach for the treatment of CRC patients harboring oncogenic PIK3CA mutations.

Keywords: Colorectal cancer, PI3KCA mutation, circLHFPL2, miR-556-5p, miR-1322, PTEN

Introduction
Class I phosphoinositide 3-kinase (PI3K) is a dimeric enzyme which phosphorylates phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) [1]. PIP3 plays a pivotal role as a second cellular messenger to activate downstream AKT signaling pathway and modulates various biological processes including cell proliferation, cell cycle, and motility [2]. PI3K contains a catalytic and a regulatory subunit [3]. The catalytic subunit consists of four isoforms including p110α, p110β, p110γ and p110δ. PI3KCA encodes p110α and is one of the most frequently mutated oncogenes in colorectal cancer (CRC) [4–6]. Mutations of PIK3CA (Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha) lead to a sustained activation of the PI3K/AKT signaling pathway in a growth factor-independent manner, promoting the growth and invasion of CRC cells [6, 7].

Most PIK3CA mutations commonly occur in two regions: an acidic cluster (E545K) in the helical domain and a histidine residue (H1047R) in the kinase domain of p110α protein [8]. E545K and H1047R are the most frequently observed p110α somatic mutations in human cancers that induce downstream AKT activation in the absence of growth factor stimulation [9]. In cells without PIK3CA mutation, receptor tyrosine kinases (RTKs) are activated by growth factors, and p110α is brought to the cell membrane through the binding of p85 and phospho-IRS1, therefore converting PIP2 to PIP3. In cancer cells harboring a p110α helical domain mutation, the p110α mutant directly binds to IRS1, thereby being recruited to the cell membrane and converting PIP2 to PIP3 without RTKs activation [7]. Dysregulation of the PI3K signaling pathway is commonly associated with tumorigenesis and drug resistance such as resistance to MEK inhibitors, while the underlying mechanism needs to be further investigated [10–12].

Circular RNAs (circRNAs) are a subset of endogenous non-coding RNAs (ncRNAs), consisting of a closed loop structure connecting the 5′ and 3′ ends [13]. CircRNAs are essential for diverse regulatory mechanisms, including competing endogenous RNAs (ceRNAs), transcriptional regulation, protein interactions and translational regulation [14–16]. In CRC, circRNAs act as sponges of miRNAs to abrogate the inhibition of miRNAs on their target genes, thereby regulating diverse biological processes, including cell growth, apoptosis, migration and invasion [17–19]. However, whether circRNAs are involved in PI3K downstream activation and drug resistance of CRC remains largely unknown.

In the present study, we reported that circLHFPL2 downregulated by PIK3CA mutation mediates FOXO3a phosphorylation and inhibits PTEN expression by sponging miR-553 and miR-1266 in CRC. We further demonstrated that circLHFPL2 is involved in PIK3CA mutation-mediated MEK inhibitor resistance by down-regulating P-gp and BCRP. Targeting circLHFPL2 could be an effective approach for the treatment of CRC patients harboring tumor mutations of this gene.

Materials and methods
Patients and samples
We collected tumor tissues from CRC patients diagnosed between January 2014 and December 2015 in Changhai Hospital with the following inclusion criteria: 1) patients were diagnosed with CRC by using biopsy pathology; 2) pathological result was identified as adenocarcinoma, or mucinous adenocarcinoma; 3) CRC was the first and only malignant tumor without metastasis; 3) neoadjuvant therapy was not administered before surgery; 4) radical resection was performed and tumor tissue samples were obtained, snap-frozen in liquid nitrogen, and stored at −80 °C at the time of surgery; 5) sufficient clinicopathological information and successful follow-up data were collected. Preoperative characteristics consisted of age, gender, primary tumor location, body mass index (BMI), preoperative serum CEA and CA199. Postoperative pathological characteristics included tumor size, histology, T stage, N stage, tumor deposit, intravascular invasion, and perineural invasion, and were evaluated according to the seventh AJCC/TNM staging system. The mutation statuses on PIK3CA Exon-9 (E545K or E542K) and Exon-20 (H1047R or H1047L) were routinely tested.

Finally, a total of 1124 CRC cases were included in our study. Among them, 36 (3.20%) CRC cases had a PIK3CA mutation. To adjust for significant clinicopathologic covariates between PIK3CA mutation and WT groups, a propensity score matching (PSM) was implemented to reduce the possibility of selection bias by using a logistic regression model. All the variables at baseline were
used as criteria to calculate the propensity scores. PSM produced 30 cases in PIK3CA mutation and 30 cases in WT groups. All the clinicopathological characteristics in these two groups showed no statistical difference ($P \geq 0.05$; Table 1). Then, the tumor tissues from these 60 CRC cases were used for qRT-PCR to detect the expression of circLHFPL2, miR-556-5p and miR-1322, and for Western blotting or IHC staining to determine the protein levels of PTEN, P-glycoprotein (P-gp/ABCB1) and Breast cancer resistance protein (BCRP/ABCG2).

**Cell lines and cell cultures**

CRC cell lines including HCT116, DLD1, HT29, SW480, LoVo, and RKO cells were purchased from the American Type Culture Collection (ATCC, Washington, D.C., USA) and these cell lines were tested negative for mycoplasma. HCT116 cells with PIK3CA WT and PIK3CA H1047R, and DLD1 cells with PIK3CA WT and PIK3CA E545K were established as previously described [6]. All cells were cultured with McCoy’s 5A medium (Thermo, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific, Inc.), penicillin and streptomycin (100 U/ml; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator (Thermo, Waltham, USA) with 5% CO2 at 37 °C. For MEK inhibition, AZD6244 or RDEA199 was dissolved in DMSO (concentration set to 0, 0.01, 0.1, 1, 10 μM) and added into cell culture medium for 24 h.

**CircRNA sequencing**

Three batches of RNA samples were extracted from each cell lines (HCT116 cells with PIK3CA WT or E545K mutation and DLD1 cells with PIK3CA WT or H1047R mutation) and quantified by NanoDrop ND-1000 (Thermo, Waltham, USA). The RNA samples were then subjected to library construction and sequenced on Illumina HiSeq 4000 according to the guidelines of the Illumina TruSeq™ Stranded Total RNA Library Prep Kit [20]. RNA sequencing was conducted by Majorbio (Shanghai, China). Differential expression of circRNAs was analyzed by the limma package of R software. Significantly differentially expressed circRNAs were screened by $|\log_2 \text{fold change}| > 1$ and $p$ value < 0.05.

**Cell transfection and establishment of circLHFPL2 overexpressing cells**

CRC cells were seeded into 6-well plates. After cultivation to 70% confluence, miRNA mimics, small interfering RNAs (siRNAs) and corresponding controls were transfected into cells using Lipofectamine™ 3000 (L3000008; Invitrogen; Thermo, Waltham, USA) according to the manufacturer’s instructions. The siRNA sequences are shown in Additional file 1. The plasmids containing an empty vector or full-length circLHFPL2 were subcloned into pCDH-CMV-MCS-EF1-PURO lentivirus vector to establish the stable cell lines. Puromycin was used to select clone cells successfully infected with the virus, and qRT-PCR was used to confirm the expression of circLHFPL2 in the cells.

**RNA extraction and qRT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo, Waltham, USA) following the manufacturer’s guidelines [21]. cDNA was obtained through reverse transcription of total RNA with the PrimeScript RT Master Mix (Takara Bio, Inc., Dalian, China) from 300 ng of RNA. The abundance of circRNA was determined by qPCR with SYBR Premix Ex Taq II kit (Takara Bio, Inc., Dalian, China) according to the manufacturer’s instructions. GAPDH was used to normalize the expression of circLHFPL2 and PTEN (Supplementary Fig. 4), and U6 was used to normalize the expression of miR-556-5p and miR-1322. The relative abundance of expression was calculated with the $2^{\Delta \Delta Ct}$ method. The sequences of all the primers are shown in Additional file 1: Table S1.

**Western blotting assay**

Total protein was isolated with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with EDTA-free protease inhibitor cocktail (cat. no. 04693159001; Roche Diagnostics GmbH). The BCA assay kit (Thermo, Waltham, USA) was used to determine the protein concentrations. Then, proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were then blocked in 5% nonfat milk for 2 h at room temperature. Primary antibodies were used to incubate the membranes at dilution ratios recommended by the manufacturers at 4°C overnight. Then, horseradish peroxidase-conjugated secondary antibodies with a dilution ratio of 1:5000 were used to incubate the membranes at room temperature for 1 h. Finally, enhanced chemiluminescence (Tanon Science and Technology, Shanghai, China) was used to develop the blots and the reaction bands were visualized in an imaging system (Tanon 4600, Tanon Science and Technology, Shanghai, China). GAPDH was used as an internal control. All the information of antibodies used in our study was shown in Additional file 2: Table S2.

**Cell viability assay**

Cell Count Kit-8 (CCK8, Takara Bio, Inc., Dalian, China) assay was used to assess the viability of CRC cells as described previously [22]. Briefly, 1.5 × 10³ cells per well were seeded into 96-well plates and maintained in medium containing 0.5% FBS. After being cultured for 1d, 2d, 3d, 4d and 5d, 10 μL CCK-8 solution was added
Table 1 Comparison of baseline characteristics of CRC cases with WT or Mutant PIK3CA status before and after propensity score matching (PSM)

| Characteristic                        | Before PSM | Mutant cohort | After PSM | Mutant cohort | P value |
|---------------------------------------|------------|---------------|-----------|---------------|---------|
|                                       | WT cohort  | Mutant cohort |           |               |         |
|                                       | (n=1088)   | (n=36)        |           |               |         |
| Agea (years)                          | 62.0 (54.0‑69.0) | 65.0 (51.0‑73.2) | 0.055     | 59.6±14.0     | 63.4±13.2 | 0.2893 |
| Sex                                   |            |               |           |               | 0.081   | 0.7961 |
|                                       | Male       | 685           | 17        | 13            | 15      |         |
|                                       | Female     | 403           | 19        | 17            | 15      |         |
| Tumor location                        |            |               |           |               | <0.001  | 0.607  |
|                                       | Rectum     | 577           | 9         | 6             | 9       |         |
|                                       | Left colon | 288           | 7         | 5             | 6       |         |
|                                       | Right colon| 223           | 20        | 19            | 15      |         |
| BMI (kg/m²)                           |            |               |           |               | 0.015   | 0.289  |
|                                       | <18.5      | 43            | 5         | 1             | 4       |         |
|                                       | 18.5‑23.9  | 588           | 17        | 19            | 14      |         |
|                                       | >23.9      | 457           | 14        | 10            | 12      |         |
| CEA level (U/ml)                      |            |               |           |               | 0.323   | 1.000  |
|                                       | ≥5         | 375           | 11        | 8             | 7       |         |
|                                       | <5         | 674           | 22        | 19            | 20      |         |
|                                       | Unknown    | 39            | 3         | 3             | 3       |         |
| CA199 level (U/ml)                    |            |               |           |               | <0.001  | 0.510  |
|                                       | ≥37        | 168           | 16        | 7             | 11      |         |
|                                       | <37        | 882           | 17        | 20            | 16      |         |
|                                       | Unknown    | 38            | 3         | 3             | 3       |         |
| Tumor sizea (cm)                      |            |               |           |               | 0.421   | 0.572  |
|                                       | 4.0 (3.0‑5.5) | 4.8 (3.0‑6.0) |           | 4.8 (4.0‑6.0)| 4.5 (3.1‑6.0)| 0.785  |
| Histology                             |            |               |           |               | <0.001  |         |
|                                       | Well/moderately differentiated Adenocarcinoma | 901 | 20 | 21 | 19 |         |
|                                       | Poorly differentiated Adenocarcinoma/Mucinous adenocarcinoma | 187 | 16 | 9 | 11 |         |
| T stage                               |            |               |           |               | 0.306   | 0.998  |
| T1                                    | 50         | 1             | 0         | 1             |         |
| T2                                    | 219        | 2             | 0         | 2             |         |
| T3                                    | 695        | 26            | 24        | 22            |         |
| T4                                    | 124        | 7             | 6         | 5             |         |
| Nstage                                |            |               |           |               | 0.016   | 0.135  |
| N0                                    | 633        | 12            | 15        | 12            |         |
| N1                                    | 312        | 16            | 7         | 14            |         |
| N2                                    | 143        | 8             | 8         | 4             |         |
| Tumor deposit                         |            |               |           |               | 0.004   | 1.000  |
| Positive                              | 150        | 12            | 6         | 6             |         |
| Negative                              | 928        | 24            | 24        | 24            |         |
| Unknown                               | 10         | 0             | 0         | 0             |         |
| Intravascular invasion                |            |               |           |               | 0.001   | 1.000  |
| Positive                              | 82         | 9             | 5         | 4             |         |
| Negative                              | 996        | 27            | 25        | 26            |         |
| Unknown                               | 10         | 0             | 0         | 0             |         |
| Perineural invasion                   |            |               |           |               | 0.055   | 1.000  |
| Positive                              | 109        | 8             | 5         | 4             |         |
| Negative                              | 969        | 28            | 25        | 26            |         |
| Unknown                               | 3          | 0             | 0         | 0             |         |

* Except these, other values were summarized as frequencies and percentages
were designed using the same approach. pmirGLO luciferase reporter plasmids were designed with or without a 3′-untranslated region binding site for miR-556-5p and miR-1322. PTEN-Mut were designed with or without a 3′-untranslated region binding site for miR-556-5p and miR-1322.

**Colony formation assay**
The treated CRC cells were digested and seeded into 6-well plates at a density of 1 × 10^4 cells/well. Then, cells were cultured in medium containing 0.5% FBS and maintained in an incubator with 5% CO₂ at 37°C for 14 d. Subsequently, cells were washed with PBS and fixed with paraformaldehyde for 10 min at room temperature. The fixed cells were stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 20 min.

**Cell apoptosis assay**
The treated cells were cultured in medium containing 0.5% FBS and maintained in an incubator with 5% CO₂ at 37°C for 48 h. Then, cells were digested and washed twice with PBS and resuspended in buffer. Next, Annexin V-fluorescein isothiocyanate apoptosis detection kit (Becton, Dickinson and Company, New Jersey, USA) was utilized according to the manufacturer’s instructions. The cell apoptosis analysis was conducted with flow cytometry using the BD FACSVantage™ SE System (Becton, Dickinson and Company, New Jersey, USA).

**Luciferase reporter assay**
The pmirGLO luciferase reporter plasmids (Promega, Madison, WI, USA) of circLHFPL2-WT and circLHFPL2-Mut were designed with or without a 3′-untranslated region binding site for miR-556-5p and miR-1322. PTEN-WT and PTEN-Mut pmirGLO luciferase reporter plasmids were designed using the same approach. pmirGLO luciferase reporter plasmids were co-transfected into the cells with miRNA mimics or NC mimics using Lipofectamine 3000 Reagent (Invitrogen, Waltham, USA). Then, the Dual-Luciferase Reporter Assay Kit (Promega, Madison, USA) was used to measure the activities of Luciferase and Renilla according to the manufacturer’s instructions.

**Biotin-coupled miRNA capture**
The 3′ end of biotinylated miR-556-5p and miR-1322 mimics or control RNA (Ribio, Guangzhou, China) were transfected into 1 × 10⁶ HCT116 and DLD1 cells at a final concentration of 50 nM for 48 h before harvest. Then the cell pellet was incubated with 0.7 mL lysis buffer (5 mM MgCl₂, 100 mM KCl, 20 mM Tris [pH7.5], 0.3% NP-40, 50 U of RNase OUT (Invitrogen, Waltham, USA) and complete protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) on ice for 10 min. The biotin-coupled RNA complex was pulled down by incubating the cell lysates with streptavidin-coated magnetic beads (Thermo, Waltham, USA) and centrifugation at 10,000 × g for 10 min. The abundance of circLHFPL2 in the bound fraction was evaluated by qRT-PCR analysis.

**In vivo experiment**
The protocols of animal experiments were reviewed and approved by the Animal Care Committee of Shanghai Hospital. Six-week-old C57BL/6 nude mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China) and maintained according to the guidelines of the National Institutes of Health. Treated cells (3 × 10⁶, 100 μL) were subcutaneously injected in the left flank of the mice. Tumor size was determined every 3 days as previously reported (Length × Width² × 0.5). After 30 days, the mice were sacrificed, and the tumor weight was measured. For MEK inhibitor treatment, 10 mg/Kg of AZD6244 was injected intraperitoneally every 3 days into tumor models. Tumor size was determined every 3 days as previously reported (Length × Width² × 0.5).

**Statistical analysis**
Kaplan-Meier method was used to perform survival analysis and a log-rank test was used for comparison. Student’s t test was performed to compare the statistical difference of two groups and one-way ANOVA analysis was conducted for multiple groups. Pearson’s correlation analysis was performed between the expression levels of two groups of RNAs. Data are plotted as mean ± SEM. A significantly statistical difference was determined at P < 0.05.

**Results**
CircLHFPL2 is downregulated in PIK3CA-mutant CRC cells and tissues
Most PIK3CA mutations are clustered in two common regions, with H1047R in the kinase domain and E545K.
Fig. 1 (See legend on previous page.)
in the helical domain. It was found that the CRC ce
line HCT116 harbors a heterozygous H1047R muta-
tion, whereas DLD1 CRC cells have a heterozygou
E545K mutation (Fig. 1A). We evaluated these cell
cells in situations where either the WT or the mutan
t allele of PIK3CA was knocked out (Fig. 1A). The ce
in which the mutant allele was disrupted and the WT
t allele was intact was named ‘WT’ (Fig. 1A), whereas
cells where only the WT allele was disrupted and the mutan
t allele was intact was named ‘mutant’ (Mut, Fig.
1A). To define the expression profiles of circRNA
in PIK3CA-mutant CRC cells, circRNA sequenc-
ing by HiSeq, which captured 78,346 circRNAs, was
plemented using HCT116 cells with PIK3CA WT or
PIK3CA<sup>H1047R</sup> as well as DLD1 cells with PIK3CA WT or
PIK3CA<sup>E545K</sup> (step1 as shown in Fig. 1B). In HCT116
cells with WT or Mut PIK3CA, 336 circRNAs were
upregulated and 163 circRNAs were downregulated,
while in DLD1 cells with WT or Mut PIK3CA, 692
circRNAs were upregulated and 669 circRNAs were
downregulated (step2 as shown in Fig. 1B). Thirty of
the most downregulated or upregulated circRNAs in
HCT116 and DLD1 cells with PIK3CA mutation are
shown in the heatmap (step 4 as shown in Fig. 1A, B
and C). CircLHFPL2 was the only circRNA found
among the top dysregulated circRNAs in both cell
lines (step 4 as shown in Fig. 1B, C and D). Then, we
validated the expression of circLHFPL2 in the paren-
tal, WT and Mut HCT116 cells and DLD1 cells. The
results confirmed that the expression of circLHFPL2
was significantly downregulated in HCT116 cells
with PIK3CA<sup>H1047R</sup> compared with that in the paren-
tal or HCT116 PIK3CA<sup>WT</sup> cells (Fig. 1E). Similarly, the expression of circLHFPL2 was also remarkably down-
regulated in DLD1 cells with PIK3CA<sup>E545K</sup> compared with that in the parental DLD1 cells or those with
PIK3CA<sup>WT</sup> (Fig. 1F). Then, we investigated the expres-
sion profiles of circLHFPL2 in other CRC cell lines. Figure
1E showed that circLHFPL2 was significantly downregulated in PIK3CA-mutant cell lines including
SW480 (PIK3CA<sup>H1047R</sup> cells) and LoVo (PIK3CA<sup>P449T</sup>
cells) but not in PIK3CA WT cell lines (RKO and HT29). Furthermore, we collected tumor tissues from
30 CRC patients with PIK3CA mutations and 30
patients without PIK3CA mutations, and analyzed the expression pattern of circLHFPL2 in these tissues. The patients had no significant difference in TNM stage and tumor size (Table 1). We found that expression of circLHFPL2 was significantly lower in PIK3CA-
mutant CRC tissues than that of WT PIK3CA tis-
sues (Fig. 1G). We also observed that LHFPL2 mRNA
was downregulated in PIK3CA-mutant CRC tissues
(Supplementary Fig. 5A). Moreover, survival analysis
revealed that in PIK3CA-mutant patients, those with
relatively low expression of circLHFPL2 was signifi-
cantly associated with poor overall survival (Fig. 1H).
These results demonstrate that circLHFPL2 is down-
regulated in PIK3CA-mutant CRC cell lines and tis-
sues, and downregulation of circLHFPL2 is correlated
with poor prognosis.

CircLHFPL2 is the target gene of the transcription factor Foxo3a

We next assessed the structure of circLHFPL2, which was derived from exon 4 of LHFPL2 and formed a 615 nt
circular transcript. The back-spliced junction of circL-
HFPL2 was amplified and confirmed by Sanger sequenc-
ing, which was consistent with the CircBase database
(http://www.circbase.org/) [23] (Fig. 2A). To confirm
the head-to-tail splicing of circLHFPL2, convergent primers for LHFPL2 mRNA and special divergent prim-
ers for circLHFPL2 were designed. RNA and genomic
dNA (gDNA) were isolated from HCT116 and DLD1
cells and PCR amplification was performed. The results
showed that circLHFPL2 amplified by divergent primer
was detected only in cDNA, but not detected in gDNA
(Fig. 2B). Then, the cytoplasm and nuclei were fractioned.
CircLHFPL2 was found to be enriched in the cytoplasmic
fraction, but not in the nuclei (Fig. 2C).

As previously reported, PI3K/AKT signaling pathway
was continuously activated in PIK3CA-mutant cells [7].
Therefore, we investigated whether circLHFPL2 down-
regulation in PIK3CA-mutant cells was regulated by
the activation of PI3K/AKT signaling pathway. With
the treatment of AKT agonist SC-79, the expression
of p-AKT was significantly increased in HCT116 and
DLD1 cells. Notably, circLHFPL2 and LHFPL2 expres-
sion in these two cell lines was statistically significantly
Fig. 2  (See legend on previous page.)
Overexpression of circLHFPL2 inhibited the growth of PIK3CA-mutant CRC cells. A Relative expression of circLHFPL2 determined via qRT-PCR in HCT116 PIK3CA<sup>WT</sup> or PIK3CA<sup>H1047R</sup> (left) and DLD1 PIK3CA<sup>WT</sup> or PIK3CA<sup>E545K</sup> cells (right) transfected with circLHFPL2 overexpression lentivirus. B CCK-8 assay revealed the viability of HCT116 PIK3CA<sup>WT</sup> or PIK3CA<sup>H1047R</sup> (left) and DLD1 PIK3CA<sup>WT</sup> or PIK3CA<sup>E545K</sup> cells (right) transfected with circLHFPL2 overexpression lentivirus. C Colony formation assay revealed the proliferation of HCT116 PIK3CA<sup>WT</sup> or PIK3CA<sup>H1047R</sup> (left) and DLD1 PIK3CA<sup>WT</sup> or PIK3CA<sup>E545K</sup> cells (right) transfected with circLHFPL2 overexpression lentivirus. D Apoptosis assay showed the apoptosis of HCT116 PIK3CA<sup>WT</sup> or PIK3CA<sup>H1047R</sup> (left) and DLD1 PIK3CA<sup>WT</sup> or PIK3CA<sup>E545K</sup> cells (right) transfected with circLHFPL2 overexpression lentivirus. E Western blotting showed the protein expression of BCL-2, Bax and cleaved PARP in HCT116 PIK3CA<sup>WT</sup> or PIK3CA<sup>H1047R</sup> (left) and DLD1 PIK3CA<sup>WT</sup> or PIK3CA<sup>E545K</sup> cells (right) transfected with circLHFPL2 overexpression lentivirus. Data are presented as mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01.
decreased in a concentration-dependent manner (Fig. 2D, Supplementary Fig. 5B). We predicted the transcription factor of circLHFPL2 using [24] (http://jaspar.genereg.net/) and found that the promoter region of circLHFPL2 contained 11 binding sites with Foxo3a (Fig. 2E upper panel). Interestingly, SC-79 treatment remarkably elevated the level of p-Foxo3a, indicating that activated PI3K/AKT axis may suppress the expression of circLHFPL2 by phosphorylating the transcription factor, Foxo3a (Fig. 2D). Overexpression of Foxo3a could upregulate PTEN expression in HCT116 and DLD1 cells (Supplementary Fig. 8A and B). We investigated

Fig. 4 Overexpression of circLHFPL2 inhibited the growth of PIK3CA-mutant CRC xenograft tumor. A Treated cells (3 x 10⁶, 100 μL) were subcutaneously injected into the left flank of the mice. Tumor size was determined as previously reported (Length x Width² x 0.5) every 3 days. After 30 days, the mice were sacrificed, and the tumor weight was measured. B IHC staining showed the protein expression of BCL-2, Bax, cleaved PARP in xenograft tumors. C A mouse model of lung metastases established by tail vein injection of the indicated HCT116 PIK3CAH1047R and DLD1 PIK3CAE545K cells with circLHFPL2 overexpression. Representative bioluminescence images (C, left) and bioluminescence signals (C, right) acquired at 8 weeks after injection. Data are presented as mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01.
the interaction between circLHFPL2 and Foxo3a using luciferase and ChIP assays, and three predicted binding sites of circLHFPL2 promoter and Foxo3a were identified. Foxo3a significantly increased the luciferase activity of circLHFPL2-WT, while no significant change was detected in circLHFPL2-Mut (Fig. 2E). Consistent with this result, the ChIP assay showed that Foxo3a directly occupied these three regions on circLHFPL2 promoter (Fig. 2F). Collectively, these results suggest that circLHFPL2 is mainly expressed in the cytoplasm and is suppressed by activated PI3K/AKT via phosphorylation of Foxo3a.

**Overexpression of circLHFPL2 sabotages the viability of PIK3CA-mutant cells**

Having demonstrated that the expression of circLHFPL2 was dramatically decreased in PIK3CA-mutant cells, we explored how the cells reacted to the overexpression of circLHFPL2. To this end, we overexpressed circLHFPL2 with lentivirus in HCT116 PIK3CA-WT or PIK3CA-H1047R and DLD1 PIK3CA-WT or PIK3CA-E545K cells (Fig. 3A). Then, we examined the effect of circLHFPL2 overexpression on cell proliferation using CCK-8 and colony formation assays. The results showed that overexpression of circLHFPL2 significantly suppressed cell viability, especially in PIK3CA-mut cells (Fig. 3B and C). Moreover, apoptosis assays showed that circLHFPL2 overexpression promoted apoptosis in CRC cells, especially in PIK3CA Mut cells (Fig. 3D). Western blotting revealed that anti-apoptosis protein BCL-2 was downregulated, while apoptosis-associated proteins Bax and cleaved PARP were upregulated (Fig. 3E). To further confirm the antitumor effect of circLHFPL2, we designed and synthesized two siRNAs (si-circLHFPL2–1 and si-circLHFPL2–2) targeting the back-splicing junction of circLHFPL2 to silence circLHFPL2 in HCT116 cells (Supplementary Fig. 3A). As shown in Supplementary Fig. 3B-C, silencing circLHFPL2 promotes HCT116 cell proliferation and inhibits cell apoptosis. Finally, we explored how circLHFPL2 affected the in vivo tumor growth using subcutaneous xenograft tumor model with PIK3CA Mut cells. The results showed that circLHFPL2 overexpression in HCT116 PIK3CA-H1047R and DLD1 PIK3CA-E545K cells significantly suppressed the growth of xenograft tumors (Fig. 4A). Moreover BCL-2 was downregulated while Bax and cleaved PARP were upregulated when circLHFPL2 was overexpressed (Fig. 4B). We detected the effect of linear LHFPL2 overexpression on HCT116 proliferation and found that overexpression of LHFPL2 have no effect on proliferation of HCT116 cells (Supplementary Fig. 5C-E). In the lung metastasis model, stable overexpression of circLHFPL2 in HCT116 PIK3CA-H1047R cells substantially inhibited tumor metastasis to the lung (Fig. 4C). Taken together, these results revealed that circLHFPL2 inhibits PIK3CA-mutant CRC progression both in vitro and in vivo.

**CircLHFPL2 acts as a ceRNA to sponge miR-556-5p and miR-1322**

Bioinformatics online analyses were utilized to predict the potential targets of circLHFPL2 by using Starbase and Circinteractome database. 10 predicted miRNAs related to cancer progression were selected for further analysis (Fig. 5A, Table S3). Dual-luciferase assays showed that miR-556-5p and miR-1322 were the potential downstream miRNAs binding to circLHFPL2 (Fig. 5B, supplementary Fig. 1A and B). Fluorescence in situ hybridization (FISH) showed that circLHFPL2 was localized in the cytoplasm, similar to mature miRNAs [22] (Fig. 5C). Dual-luciferase reporter and RNA pull-down assays were then implemented to confirm the interaction between circLHFPL2 and miR-556-5p and miR-1322. Cells co-transfected with pGL3-circLHFPL2 WT plasmid and miR-556-5p mimics exhibited reduced activity compared with that in the control groups (Fig. 5D). In line with this, biotinylated miR-556-5p probe effectively captured circLHFPL2 compared with control groups (Fig. 5E and J [left panel]). In addition, cells co-transfected with circLHFPL2 WT and miR-1322 mimics also showed decreased activity compared with that in the control groups (Fig. 5G). Similarly, biotinylated miR-1322 probe effectively captured circLHFPL2 relative to control groups (Fig. 5H and J [right panel]). We further evaluated the expression of miR-556-5p and miR-1322 in 30
Fig. 5 (See legend on previous page.)
CRC PIK3CA Mut tissues and 30 PIK3CA WT tissues. The results showed that miR-556-5p and miR-1322 were significantly upregulated in CRC PIK3CA-mutant tissues (Fig. 5K). qRT-PCR results showed that miR-556-5p and miR-1322 were upregulated in circLHFPL2-overexpressed transplant tumors (Supplementary Fig. 6B). We also detected miR-556-5p and miR-1322 expression after SC-79 treatment. As shown in Fig. 5L-M, miR-556-5p and miR-1322 expression in these two cell lines were statistically significantly increased in a concentration-dependent manner after SC-79 treatment. The correlation between circLHFPL2 and miR-556-5p/miR-1322 in patient tissues was analyzed. It was found that the expression of miR-556-5p or miR-1322 was negatively
associated with circLHFPL2 abundance (Fig. 5F and I). Moreover, survival analysis revealed that patients with high expression of miR-1322 and miR-556-5p were significantly associated with poor overall survival (Supplementary Fig. 6C). We detected miR-1322 and miR-556-5p expression in HCT116 and DLD1 cells transfected with Foxo3a-overexpressing plasmid (Supplementary Fig. 8A), and found that overexpression of Foxo3a significantly inhibited miR-1322 and miR-556-5p expression (Supplementary Fig. 8C). These results revealed that circLHFPL2 acts as a ceRNA to sponge miR-556-5p and miR-1322 in CRC cells.

PTEN is the downstream target gene of miR-556-5p and miR-1322

MiR-556-5p and miR-1322 play a promoting role in tumorigenesis of several types of cancers [25–27]. In line with this, our results showed the upregulation of miR-556-5p and miR-1322 in PIK3CA Mut CRC. We further explored the downstream targets of miR-556-5p and miR-1322 using Targetscan databases. PTEN was predicted as a possible downstream target of miR-556-5p and miR-1322 (Fig. 6A and Supplementary Fig. 1B and C). Dual-luciferase reporter assay was carried out to validate the association between miR-556-5p or miR-1322 and PTEN. The results showed that cells co-transfected with pGL3-PTEN WT plasmid and miR-556-5p mimics exhibited reduced activity compared with that in the control groups in 293 T cells (Fig. 6B left panel). Similarly, cells co-transfected with PTEN WT and miR-1322 mimics also exhibited lower activity relative to that in the control groups in 293 T cells (Fig. 6B right panel). Notably, protein levels of PTEN were significantly reduced after overexpression of miR-556-5p or miR-1322 in HCT116 and DLD1 cells (Fig. 6C, Supplementary Fig. 7A). Six CRC PIK3CA-mutant tissues and six PIK3CA WT tissues were selected to evaluate the expression of PTEN and circLHFPL2. The results showed a positive correlation between circLHFPL2 and PTEN expression, and both were significantly downregulated in CRC PIK3CA Mut tissues (Fig. 6D). Then, IHC staining was used to confirm the expression of PTEN in CRC PIK3CA WT and Mut tissues via TMA. The results showed that PTEN is downregulated in PIK3CA-mutant tissues (Fig. 6E). To further explore whether PTEN expression could be regulated by circLHFPL2, we measured the expression of PTEN in circLHFPL2-overexpressed cells. The results showed that PTEN protein levels were significantly upregulated in HCT116 and DLD1 cells with upregulation of circLHFPL2 (Fig. 6F), but when circLHFPL2 was silenced, its levels were downregulated (Fig. 6G). Overexpression of Foxo3a inhibited PTEN expression in HCT116 and DLD1 cells (Supplementary Fig. 8D). These results showed that circLHFPL2 upregulates PTEN expression by sponging miR-556-5p and miR-1322.

Downregulation of circLHFPL2 sustains the activation of PI3K/AKT signaling pathway by forming a regulatory loop with miR-556-5p/miR-1322/PTEN axis

To further verify whether circLHFPL2 downregulation could activate the PI3K/AKT pathway via a regulatory loop with the miR-556-5p/miR-1322/PTEN axis, we performed a series of rescue experiments. CCK-8 and colony formation assays revealed that the cell viability of HCT116 PIK3CA<sup>H1047R</sup> and DLD1 PIK3CA<sup>E545K</sup> cells was significantly inhibited after circLHFPL2 overexpression, while the cell viability was partially recovered in the presence of a combination of miR-556-5p or miR-1322 mimics or siPTEN (Fig. 7A and B, Supplementary Fig. 7B). Moreover, circLHFPL2 overexpression accelerated cell apoptosis, while the combination of miR-556-5p or miR-1322 mimics or siPTEN partially inhibited apoptosis in the HCT116 PIK3CA<sup>H1047R</sup> and DLD1 PIK3CA<sup>E545K</sup> cells (Fig. 7C). We further investigated the apoptosis-associated proteins involved in this biological process. The results showed that circLHFPL2 overexpression significantly decreased anti-apoptosis protein BCL-2 and increased PTEN, as well as apoptosis-associated proteins Bax and cleaved PARP, while these effects were partially recovered with the combination of miR-556-5p/miR-1322mimics or siPTEN (Fig. 7D). We isolated primary cells from a colon cancer tissue harboring PIK3CA E545K mutation and further confirmed that circLHFPL2 inhibits CRC progression by downregulating PTEN (Supplementary Fig. 2). Collectively, these
Fig. 7 (See legend on previous page.)
results indicated that downregulation of circLHFPL2 sustains activation of PI3K/AKT pathway by regulating miR-556-5p/miR-1322/PTEN axis.

**Overexpression of CircLHFPL2 overcomes MEK inhibition resistance in CRC with PIK3CA mutation**

In our previous study, we found that targeting PI3K overcomes P-gp- and BCRP-mediated cancer multidrug resistance (MDR) in cancers [11]. PI3K activation leads to P-gp and BCRP overexpression and decreases the sensitivity of colon cancer cells to a MEK inhibitor [12]. As shown in the above data, overexpression of circLHFPL2 decreased activation of PI3K by upregulating PTEN, suggesting that circLHFPL2 may be a potential target that could overcome P-gp- and BCRP-mediated MDR of colon cancer with PIK3CA mutation. Thus, P-gp and BCRP expression in colon cancer cells with PIK3CA mutation was detected by Western blotting analysis. As shown in Fig. 8A, P-gp and BCRP were upregulated in HCT116 PIK3CAH1047R and DLD1 PIK3CAE545K cells compared with PIK3CAWT cells, whereas P-gp and BCRP were decreased in circLHFPL2 overexpression HCT116 and DLD1, especially in PIK3CA-mutant cells. Moreover, the protein levels of P-gp and BCRP were found to be significantly higher in colon cancer tissues with PIK3CA mutations than in PIK3CA WT tissues (Fig. 8B-C). Colon cancer cells with or without PIK3CA mutation and with or without circLHFPL2 overexpression were treated with MEK inhibitors AZD6244 or RDEA119 for 24h. Compared to the HCT116 and DLD1 PIK3CA-mutant cells, the cell viability of the WT cells was greatly reduced upon treatment with AZD6244 or RDEA119, indicating that PIK3CA mutation enhanced drug resistance of colon cancer cells (Fig. 8D). Interestingly, HCT116 and DLD1 cell lines with or without circLHFPL2 overexpression were more sensitive to AZD6244 and RDEA119, especially in PIK3CA-mutant cells (Fig. 8D). Moreover, overexpression of circLHFPL2 enhanced the antitumor effect of AZD6244 in PIK3CA-mutant tumors (Fig. 8E-F). These results suggest that PIK3CA mutation enhanced MEK inhibitor resistance, which can be reversed by circLHFPL2.

**Discussion**

PIK3CA is one of the most frequently mutated oncogenic genes and plays an important role in tumorigenesis and drug resistance [5]. Recent studies have demonstrated that mutant PIK3CA can activate the PI3K/AKT signaling pathway without activating growth factors [7]. Samuels et al. demonstrated that PI3K/AKT signaling pathway was activated in cells with mutant PIK3CA at 1% FBS concentration, but was not activated in cells with WT PIK3CA [6]. Furthermore, mutant PIK3CA could gain functions independent of growth factor stimulation, by directly binding to IRS1 [7]. In this study, we revealed a novel regulatory network involving the activation of AKT and its downstream targets in a growth factor-independent manner in mutant PIK3CA CRC. Through circRNA sequencing, we identified that circLHFPL2 was downregulated in both HCT116 PIK3CAH1047R and DLD1 PIK3CAE545K cell lines, which was also observed in PIK3CA-mutant CRC primary cells and tissues. The downregulation of circLHFPL2 was correlated with poor prognosis. Furthermore, we found that circLHFPL2 downregulation was induced by activation of PI3K/AKT signaling pathway caused by PIK3CA mutation mediated by FOXO3a phosphorylation, which in turn sustained the activation of PI3K/AKT signaling pathway via a regulatory loop with miR-556-5p/miR-1322/PTEN axis. In addition, our data demonstrate that circLHFPL2 promotes the sensitivity of CRC cells to MEK inhibitors by downregulating the expression of P-gp and BCRP, particularly in PIK3CA-mutant cells. To the best of our knowledge, this study for the first time revealed a novel mechanism linking circRNA and mutant PIK3CA in continuously activating AKT signaling in a growth factor-independent manner. More importantly, we identified circLHFPL2 as a new biomarker for MEK inhibitor therapy, which has important clinical implications for the treatment of CRC with PIK3CA mutation [28]. Low circLHFPL2 expression is related to poor prognosis of CRC patients, and therefore an independent external cohort needs to further validate that circLHFPL2 could serve as a prognostic biomarker for CRC diagnosis.

CircLHFPL2 is a 615 nt circular transcript originated from exon 4 of LHFPL2. LHFPL2 is a member...
Fig. 8 (See legend on previous page.)
of the lipoma high mobility group protein isoform I-C (HMGIC) fusion partner (LHFP) gene family, which encodes a tetra-transmembrane protein [29]. LHFL2 has been implicated in diverse physiological functions, including long-term proliferation of leukemic cells, coronary heart disease and infertility [29–31]. The expression profiles and functional roles of LHFL2-formed circRNA on CRC progression have not yet been reported. CircLHFL2 overexpression suppressed CRC cell proliferation and weakened the viability of PIK3CA-mutant cells, indicating that circLHFL2 plays a crucial role in the development of PIK3CA-mutant tumors.

We also revealed the mechanism of circLHFL2 downregulation in CRC with PIK3CA mutations. FOXO3a belongs to the FOXO subfamily that acts as a forkhead transcription factor regulating various biological processes, such as proliferation, apoptosis, cell cycle and tumorigenesis [32]. Growing evidence supports that FOXO3a acts as a tumor suppressor in cancers by regulating downstream signaling targets through transcriptional modification [33, 34]. FOXO3a inactivation is observed in various cancers and is mainly caused by mutation of FOXO3a gene or cytoplasmic sequestration of FOXO3a protein induced by post-translational modification [32]. Foxo3a could be phosphorylated by activated PI3K/AKT signaling pathway, and the phosphorylated Foxo3a can bind to 14–3–3 protein, which leads to nuclear export and ubiquitination of Foxo3a, and thus inhibiting the transcription of target genes [35]. Here, we identified Foxo3a as the transcription factor of circLHFL2. Consistent with the previous study, we found that Foxo3a maintains a high phosphorylation state in cells with PIK3CA mutations, which is caused by persistently activated Akt, and explains how PIK3CA mutation downregulates circLHFL2 [36].

PTEN, namely phosphatase and tensin homologue, is an important tumor suppressor in various cancers including ovarian cancer, melanoma, gastric cancer,
breast cancer, prostate cancer and colorectal cancer. PTEN inhibits proliferation and promotes apoptosis of cancer cells [37]. PTEN dephosphorylates PI3K to PI4,2, thus abrogating PI3K/AKT activity and inhibiting the progression of cancers [38, 39]. In cancer cells, PTEN is inactivated by multiple mechanisms, including mutations, promoter hypermethylation, protein degradation and subcellular mislocalization [39]. Although PTEN is demonstrated to modulate PI3K/AKT activity, the regulatory role of mutant PIK3CA on PTEN remains an enigma. Here, we revealed a novel regulatory mechanism of mutant PIK3CA on PTEN. We found that circLHFPL2 could sponge miR-556-5p and miR-1322 in PIK3CA-mutant cells, thus regulating the expression of PTEN. Collectively, circLHFPL2 downregulation sustained the activation of PI3K/Akt signaling pathway via a regulatory loop with miR-556-5p/miR-1322/PTEN axis in PI3KCA mutant CRC.

Despite considerable efforts, the clinical outcome of treatments for solid tumors has been challenging [40–42]. Reasons include drug resistance such as that resulting from the dysregulation PI3K/Akt signaling pathway and PTEN suppression, and more. As reported, activated PI3K leads to P-gp and BCRP expression and increases the sensitivity of CRC cells to MEK inhibitor [11, 12]. Interestingly, in our study, overexpression of circLHFPL2 could inhibit the expression of P-gp and BCRP and overcome PI3KCA mutation-mediated MEK inhibitor resistance in CRC cells. In our previous study, we found that PI3K promoted drug resistance by upregulating P-gp and BCRP in human breast cancer [11]. Thus, we assumed that circLHFPL2 may inhibit P-gp and BCRP expression by regulating PI3K. Our study indicated that circLHFPL2 may serve as a potential therapy target for regulating PIK3CA mutation-mediated drug resistance. However, limited by the in vivo and in vitro models we used, the function of circLHFPL2 on regulating MEK inhibitor in the treatment of CRC patients needs to be further investigated from the perspective of clinical treatment in the future.

**Conclusion**

In summary, our study revealed a new mechanism underlying PIK3CA mutation in promoting cancer progression, as well as the function of circLHFPL2 regulated by PI3K/Akt signaling pathway in CRC. In addition, we demonstrated that circLHFPL2 regulates the sensitivity of CRC cells to MEK inhibitor and could be a biomarker for MEK inhibitor treatment in patients whose tumors harbor PIK3CA mutations (Fig. 9). Future experiments may extend our study to a large panel of CRC cell lines, patient-derived xenografts and transgenic PIK3CA-mutant mouse models.

**Abbreviations**

CRC: Colorectal cancer; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PI3K: Class I phosphoinositide 3-kinase; PTEN: Phosphatase and tensin homolog; LHFPL2: LHFPL tetraspan subfamily member 2; circRNAs: Circular RNAs; miRNA: microRNA; qRT-PCR: Quantitative RT-PCR; FISH: Fluorescence in situ hybridization; MEK: Mitogen-activated protein kinase; ceRNA: Competing endogenous RNAs; BCRP/ABCG2: Breast cancer resistance protein; P-gp/ABCB1: P-glycoprotein; TMA: Tissue microarray.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12943-022-01531-x.
Additional file 11: Supplementary Fig. 8. The effect of overexpression of FOXO3a on circHFL2, miR-556-Sp, miR-1322 and PTEN expression. (A) Western blot detected the FOXO3a expression in HCT116 and DLD1 cells transfected with FOXO3a overexpression plasmids. (B) qRT-PCR detected the circHFL2 expression in HCT116 and DLD1 cells transfected with FOXO3a overexpression plasmids. (C) qRT-PCR detected the miR-556-Sp and miR-1322 expression in HCT116 and DLD1 cells transfected with FOXO3a overexpression plasmids. (D) Western blot detected the PTEN expression in HCT116 and DLD1 cells transfected with FOXO3a overexpression plasmids.

Acknowledgements
Not applicable.

Authors’ contributions
C.X.D. C.J.D. Z.N.X and Z.Y.Y. performed all the experiments. C.J.D. Z.N.X. and Z.Z.Q. provided human specimens, clinical information and data analysis. Z.Y. Y.Q.Z. Y.S.J. C.Z.Q B.W.F. and Q. M provide support with experimental materials and techniques. Z.S.C., Z.Y.Y. and G.Y. designed the research and wrote the manuscript. The authors read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (Grant No. 81772567, No. 81972290), the Outstanding Clinical Discipline Project of Shanghai Pudong (Grant No. PWYgy2018–02), and the Clinical Research (Grant No. PWYgy2018–02), and the Clinical Research Project of Shanghai Municipal Health Commission (Grant No. 2019YQ0269).

Availability of data and materials
For all data requests, please contact the corresponding author.

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of the Changhai Hospital Affiliated to the Second Military Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 4 June 2021 Accepted: 5 February 2022 Published online: 26 May 2022

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