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ORIGINAL RESEARCH

Inhibition of miR-10a-5p suppresses cholangiocarcinoma cell growth through downregulation of Akt pathway

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Backgrounds: Cholangiocarcinoma (CCA) is epithelial cell malignancy with very poor prognosis. A lot of patients were diagnosed at advanced stage of CCA and no risk factors were identified. There are limited treatment options available for the management of CCA patients. It is urgent to develop effective targeted therapies for the treatment of CCA. miRNAs are small noncoding RNAs that negatively regulate the target genes. In this study, we investigated the role and mechanism of miR-10a-5p in CCA.

Methods: Human CCA cell lines (CCLP1 and SG-231) were transfected with miR-10a-5p mimic or miR-10a-5p inhibitor. qRT-PCR was performed to detect the miR-10a-5p level. Proliferation, colony formation, and apoptosis were analyzed. Luciferase reporter assay was used to explore the targeting of miR-10a-5p on PTEN. For in vivo tumorigenesis assay, CCLP1 cells with stable knockdown of miR-10a-5p or control CCLP1 cells were injected subcutaneously into the flank of the SCID mice and animals were monitored for tumor growth.

Results: miR-10a-5p expression was significantly upregulated in human CCA cell lines (CCLP1 and SG-231). Inhibition of miR-10a-5p significantly suppressed the proliferation and induced apoptosis in CCLP1 and SG-231. PTEN is a direct target of miR-10a-5p in CCA cells.

Conclusion: Inhibition of miR-10a-5p can decrease CCA cells growth by downregulation of Akt pathway. These results indicate that miR-10a-5p may serve as a potential target for the treatment of CCA and help to develop effective therapeutic strategies.

Keywords: miR-10a-5p, cholangiocarcinoma, PTEN, Akt, liver, proliferation

Introduction

Cholangiocarcinoma (CCA) is the second most common primary liver malignancy.[1] CCA represents a diverse group of epithelial cell malignancy that develops along the biliary tract.[2,3] CCA are classified into intrahepatic CCA (iCCA), perihilar CCA (pCCA), and distal CCA (dCCA) depending on their site of origin.[4] Different types of CCA have different features and require specific treatments. Primary sclerosing cholangitis is considered to be the principal risk factor for CCA.[5] Other risk factors include hepatitis C virus, human immunodeficiency virus, liver cirrhosis, and diabetes.[6] However, in most CCAs, no risk factors are identified. The incidence of ICC in the US continues to rise. Between 1973 and 2012, the reported US incidence of ICC increased from 0.44 to 1.18 cases per 100,000.[7] Patients with CCA often present symptoms with biliary obstruction or non-specific abdominal pain, a high proportion of patients were diagnosed at advanced stage of CCA.[8] At early stage, curative options are available in the form of surgical resection and/or liver transplantation.[9] The most frequently used treatment modality is chemotherapy. Due to high rate of recurrence after liver

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transplantation, distant metastasis and invasion, as well as the chemoresistance, CCA patients represent a very poor prognosis. The average 5-year survival rate for CCA patients is 5%–10%.[10] It is urgent to develop new specific effective targeted therapies for the treatment of CCA.

miRNAs are small noncoding RNAs which are short single-stranded molecules about 21–23 nucleotides in length.[11] miRNAs regulate gene expression at post transcriptional level. miRNAs inhibit the target genes expression by binding to 3′ untranslated regions (3′UTRs) of target mRNAs which cause mRNA degradation and destabilization.[12] miRNAs play important roles in a broad range of biological processes, such as embryonic development,[13] apoptosis,[14] stem cell differentiation,[15] hematopoiesis,[16] and immune response.[17] Dysregulation of miRNA expression has been reported in cancer, including CCA. For example, miR-29a has emerged as a tumor suppressor, miR-29a level was found significantly decreased in both CCA tissues and tumor cell lines.[18] miR-34a was rhythmically expressed in CCA cells. Inhibition of miR-34a decreased proliferation, migration, and invasion in CCA cells.[19] miR-21 and miR-221 levels significantly upregulated in CCA serum. Circulating plasma levels of miR-21 and miR-221 can serve as a diagnostic and prognostic biomarkers for CCA.[20,21]

miR-10 family including miR-10a and miR-10b has attracted attention because of its conservation and the position of the miR-10 genes within the Homeobox (HOX) clusters.[22] Hox genes are a group of evolutionarily conserved genes that encode a class of important transcription factors that regulate early developmental morphogenetic processes and continue to be expressed into adulthood.[23] Hox genes organized into four distinct clusters. These clusters, labeled HOXA, HOXB, HOXC, and HOXD, are located on chromosomes 7p14, 17q21, 12q13, and 2q31.[23] miR-10a was located within the HOX B cluster on 17q21 and miR-10b was located at HOX D cluster 2q31.[24] miR-10 family members are deregulated in numerous types of cancers including uterine sarcomas,[25] breast cancer,[26] and hepatocellular carcinoma (HCC).[27] miR-10a has been reported to be associated with liver regeneration,[28] regulates human mesangial cells proliferation and chemokine expression by targeting IL-8.[29] Plasma miR-10a levels were decreased in patients with coronary artery disease (CAD) and negatively associated with the presence and severity of CAD.[30] miR-10a serves as a switch to control miR-10a-NF-kB regulatory circuit that promotes the excessive secretion of NF-κB-mediated inflammatory cytokines and the proliferation and migration of fibroblast-like synoviocytes of rheumatoid arthritis (RA).[31] miR-10a-5p is overexpressed in human pancreatic ductal adenocarcinoma (PDAC) and acts as an oncogene to promote the metastatic behavior of PDAC cells.[32] Abnormal high expression of miR-10a was also found in patients with acute myeloid leukemia (AML). miR-10a promotes proliferation of immature blood progenitors and repression of mature blood cell differentiation and maturation in AML.[33]

The expression of miR-10 was upregulated in CCA.[34] However, the function of miR-10a-5p in CCA is largely unknown. In the present study, we explored the role of miR-10a-5p in CCA. We found that PTEN is a direct target of miR-10a-5p in CCA cell lines. Inhibition of miR-10a-5p suppressed proliferation and promoted apoptosis in CCA cells through downregulation Akt pathway.

Methods

Cell culture

Human intrahepatic bile duct epithelial cell line HIBEC was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human CCA cell lines CCLP1 and SG-231 were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, People's Republic of China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, L-glutamine, and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). All cells were maintained in a 37°C humidified incubator with 5% CO_2.

Transfections

CCLP1 and SG-231 cells were seeded in six-well plate and transfected with scramble control or miR-10a-5p mimic or miR-10a-5p inhibitor GenePharma (Shanghai, People's Republic of China) using Oligofectamine reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Final concentration of scramble or miR-10a-5p mimic or miR-10a-5p inhibitor is 100 nM. At the indicated time point, cells samples were collected.

qRT-PCR

Total RNA was extracted from cells using Trizol (Thermo Fisher Scientific). Reverse transcription was performed using the miScript RT Kit (TaKaRa, Dalian, People's Republic of China). qRT-PCR was performed using miScript SYBR Green PCR Kit (Qiagen NV, Venlo, the Netherlands) on a C1000 thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primers of miR-10a-5p and U6 were obtained from Qiagen NV. U6 was used as an internal control.

Cell proliferation assay

Proliferation assays were conducted using WST-1 assay (Beyotime, Shanghai, People's Republic of China). After CCLP1 and SG-231 cells were transfected with miR-10-a-5p mimic or miR-10a-5p inhibitor or scramble control for 6 hours, cells were seeded in 96-well plates (2,000 cells/well). At 0, 24, 48, and 72 hours, culture medium was removed and 100 µL fresh medium containing 10 µL of WST-1 reagents was added into the wells. After 2–3 hours, the absorbance was measured at 450 nm by using ELISA Microplate Reader (Biocompare, San Francisco, CA, USA).

Western blot analysis

Total protein was extracted from cells using a protein extraction kit (Beyotime). Protein concentrations were measured using the BCA Protein Assay Kit (Beyotime). Protein fractions were separated on SDS-PAGE gel electrophoresis (Bio-Rad Laboratories Inc.) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories Inc.). After blocking in 5% skim milk in PBS for 1 hour at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies against PARP, cleaved caspase-3, PTEN, p-Akt (ser473), and Akt were obtained from Cell Signaling Technology (Danvers, MA, USA). Primary antibody against β-actin was obtained from Abcam (Cambridge, MA, USA). Secondary antibodies IRDye800CW Goat anti-Mouse IgG and IRDye800CW Goat anti-Rabbit IgG were obtained from LI-COR (LI-COR Biosciences, Lincoln, NE, USA). Western bolt images were detecting by using Li-COR Odyssey 9120 Imaging System (LI-COR Biosciences).

Luciferase reporter assays

PTEN 3′-UTR was obtained from GeneCopoeia (Rockville, MD, USA). We mutated two nucleotides of the PTEN 3′-UTR by using Site-Directed Mutagenesis kit (Stratagene, Shanghai, People's Republic of China). These vectors also express the Renilla luciferase serving as internal controls for the dual-luciferase assay. CCLP1 and SG231 were co-transfected with miR-10a-5p mimic (100 nM) or scramble (100 nM) with PTEN 3′-UTR or its mutant (Mut) using lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). After 48 hours of transfection, the luciferase activity was measured using the dual luciferase reporter assay kit (Promega Corporation, Madison, WI, USA).

Colony formation assay

Lentiviral plasmid vector expresses miR-10a-5p inhibitor (LV-miR-10-5p-inhibitor) and scramble control lentivirus vector (LV-con) were obtained from ABM Industries Inc. (New York, NY, USA). We established CCLP1 cell line with stable knockdown of miR-10a-5p by transfecting cells with LV-miR-10-5p-inhibitor. The control CCLP1 cells were transfected with LV-con. Cells were seeded in 10 cm dishes at 2,000 cells/dish and cultured for 14 days. After fixation with methanol for 20 minutes, the colonies were stained with 0.1% crystal violet.

Mouse xenograft model

For tumorigenesis assays, 6 weeks old, male SCID mice were purchased from Wei Tong Li Hua Experimental Animal Technology Co., Ltd (Beijing, People's Republic of China) (n=3). In total, 1×10^6 miR-10a-5p stable knockdown CCLP1 cells (LV-miR-10a-5p-inhibitor) or control CCLP1 cells (LV-con) were injected subcutaneously into the flank of the mice. Mice were observed for 30 days for tumor formation. Tumor diameters are measured with digital calipers, and the tumor volume in mm^3 is calculated by the formula: Volume = $(\text{width})^2 \times \text{length} \div 2$. All animal studies were approved by the Ethics Committee of Fudan University Pudong Medical Center. The handling of the mice and all experimental procedures were carried out in strict accordance with Fudan University Guidelines for the Care and Use of Laboratory Animals.

Statistical analysis

Data represent the mean ± SD. Experiments were repeated at least three times. Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA along with Bonferroni adjustment and Student's *t*-test were used to evaluate the differences between groups. A P-value < 0.05 was considered statistically significant.

Results

Inhibition of miR-10a-5p suppresses proliferation and promotes apoptosis in CCA cells

We evaluated the expression of miR-10a-5p in human intrahepatic bile duct epithelial cell line HIBEC and human CCA cell lines (CCLP1 and SG-231) by qRT-PCR analysis. Results showed that miR-10a-5p was upregulated significantly in CCA cells compared with HIBEC (Figure 1A). To evaluate the role of miR-10a-5p on CCA cells growth, human CCA cell lines CCLP1 and SG-231 were transfected with miR-10a-5p mimic or miR-10a-5p inhibitor or scramble control. The expression of miR-10a-5p was determined by qRT-PCR. As shown in Figure 1B, compared with scramble control,

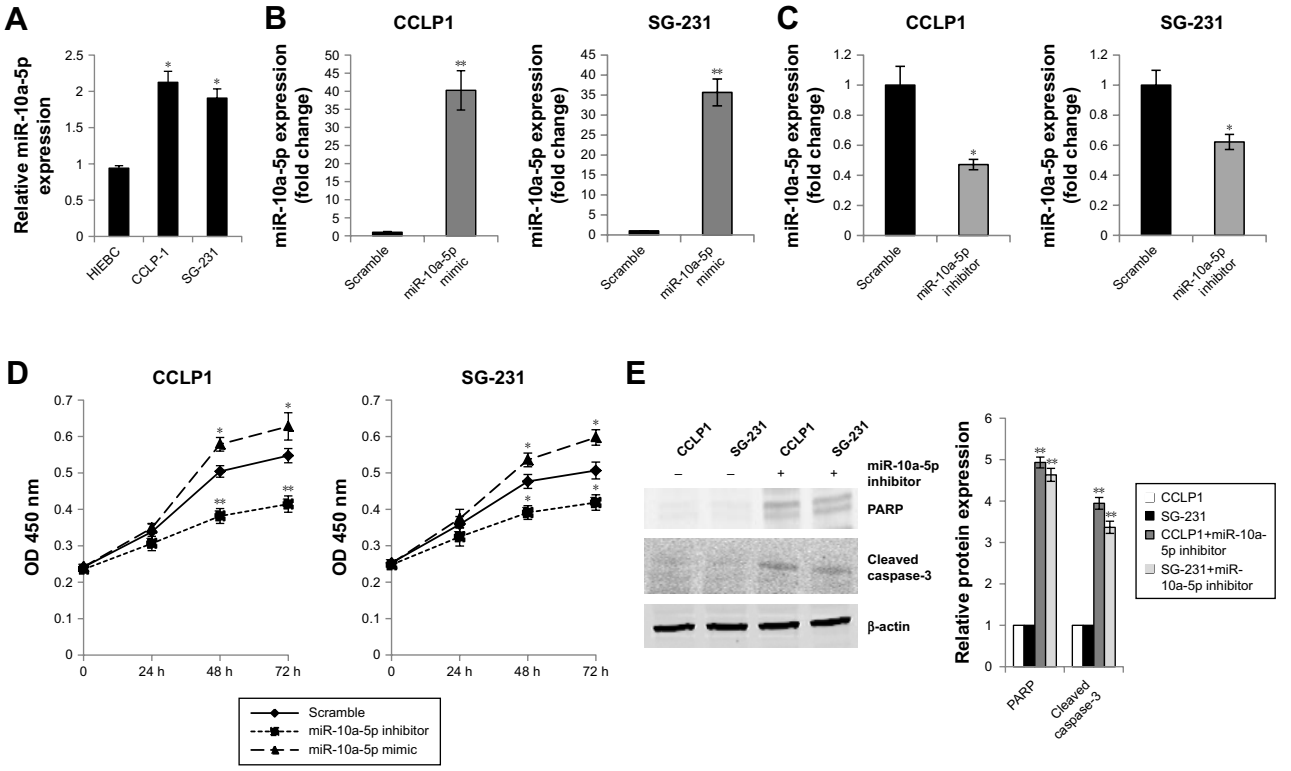

Figure 1 Inhibition of miR-10a-5p suppresses CCA cell proliferation and induces apoptosis in vitro.
Notes: (**A**) The levels of miR-10a-5p in human intrahepatic bile duct epithelial cell line HIBEC and human CCA cell lines (CCLP1 and SG231) were determined by qRT-PCR analysis. (**B**) miR-10a-5p expression was determined by qRT-PCR in CCLP1 and SG-231 cells post transfection of miR-10a-5p mimic or scramble control for 72 hours. (**C**) miR-10a-5p expression was determined by qRT-PCR in CCLP1 and SG-231 cells post transfection of miR-10a-5p inhibitor or scramble control for 72 hours. (**D**) The proliferation of CCLP1 and SG-231 cells was measured by using WST-1 analysis. (**E**) Western blot analysis of the protein levels of PARP and cleaved caspase-3. β-actin was used as an internal control. Quantifications of relative protein levels are shown at the right panel. Data were expressed as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$.
Abbreviation: CCA, cholangiocarcinoma.

transfection of miR-10a-5p mimic for 72 hours led to a dramatic increase expression of miR-10a-5p in both CCLP1 and SG231 cells, whereas transfection of miR-10a-5p inhibitor for 72 hours led to a significant inhibition of the miR-10a-5p level in these cells (Figure 1C). Cell viability was measured using WST-1 assay. As shown in Figure 1D, upregulated miR-10a-5p level by miR-10a-5p mimic significantly increased the proliferation in both of CCLP1 and SG-231 cells, whereas a significant decrease in cell viability was detected when cells transfected with miR-10a-5p inhibitor compared with scramble control. Western blot analysis revealed that the cleaved PARP and cleaved caspase-3 were significantly increased in CCLP1 and SG-231 cells transfected with miR-10a-5p inhibitor (Figure 1E). These results indicated that miR-10a-5p promoted CCA cells proliferation, while inhibition of miR-10a-5p suppressed cell growth and induced apoptosis in CCA cells.

PTEN is a direct target of miR-10a-5p in CCA cells

To explore the tumor suppressive mechanism of miR-10a-5p inhibition, the potential target genes of miR-10a-5p were analyzed using miRNA target prediction programs TargetScan (http://www.targetscan.org). There are 287 transcripts with conserved sites, containing a total of 302 conserved sites and 99 poorly conserved sites. The predicted targets of human miR-10a-5p are shown in Table S1. We found that there was a predicted miR-10a-5p binding site in the 3′-UTR of PTEN (PTEN, phosphatase, and tensin homologue deleted on chromosome ten) (Figure 2A). To determine whether PTEN was regulated by miR-10a-5p, CCLP1 and SG-231 cells were transfected with miR-10a-5p inhibitor, Western blot analysis showed that inhibition of miR-10a-5p significantly upregulated the protein levels of PTEN (Figure 2B) and decreased the expression of p-Akt (ser473) (Figure 2C). To further verify whether PTEN is a direct target of miR-10a-5p, we generated PTEN reporter construct containing 3′-UTR with mutations of miR-10a-5p binding site (indicated in Figure 2A). CCLP1 and SG-231 cells were transfected with wild type or Mut PTEN 3′-UTR and miR-10a-5p mimic, luciferase reporter assay showed that miR-10a-5p mimic remarkably decreased the 3′-UTR luciferase reporter activity of PTEN, this effect was abolished when miR-10a-5p binding site was mutated (Figure 2D). These findings

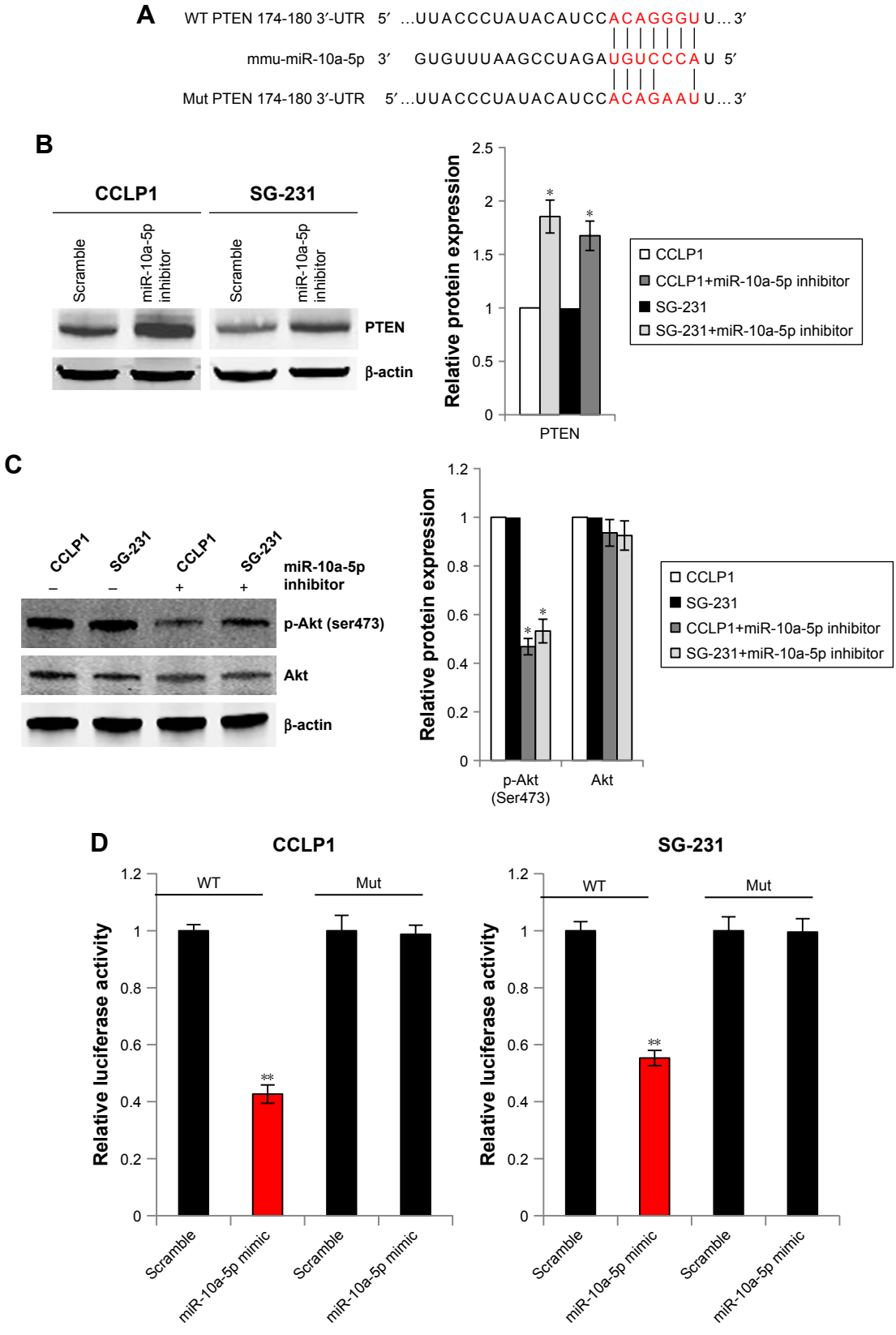

Figure 2 PTEN was a direct target of miR-10a-5p in CCA.

Notes: (**A**) The 3′-UTR of PTEN contained a predicted miR-10a-5p binding site. Mutations were generated on the two nucleotides of the PTEN 3′-UTR as indicated. (**B**) CCLP1 and SG-231 were transfected with miR-10a-5p inhibitor or scramble control for 48 hours, protein levels of PTEN were determined by Western blot analysis. Quantifications of relative protein levels are shown at the right panel. (**C**) Western blot analysis of p-Akt (ser473) and total Akt. Quantifications of relative protein levels are shown at the right panel. (**D**) Relative luciferase activity in CCLP1 and SG-231 cells co-transfected with WT or Mut PTEN 3′-UTR and miR-10a-5p mimic or scramble control. Red bar indicates statistical difference. Data were expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Abbreviations: CCA, cholangiocarcinoma; 3′-UTR, 3′-untranslated regions; Mut, mutation; WT, wild type.

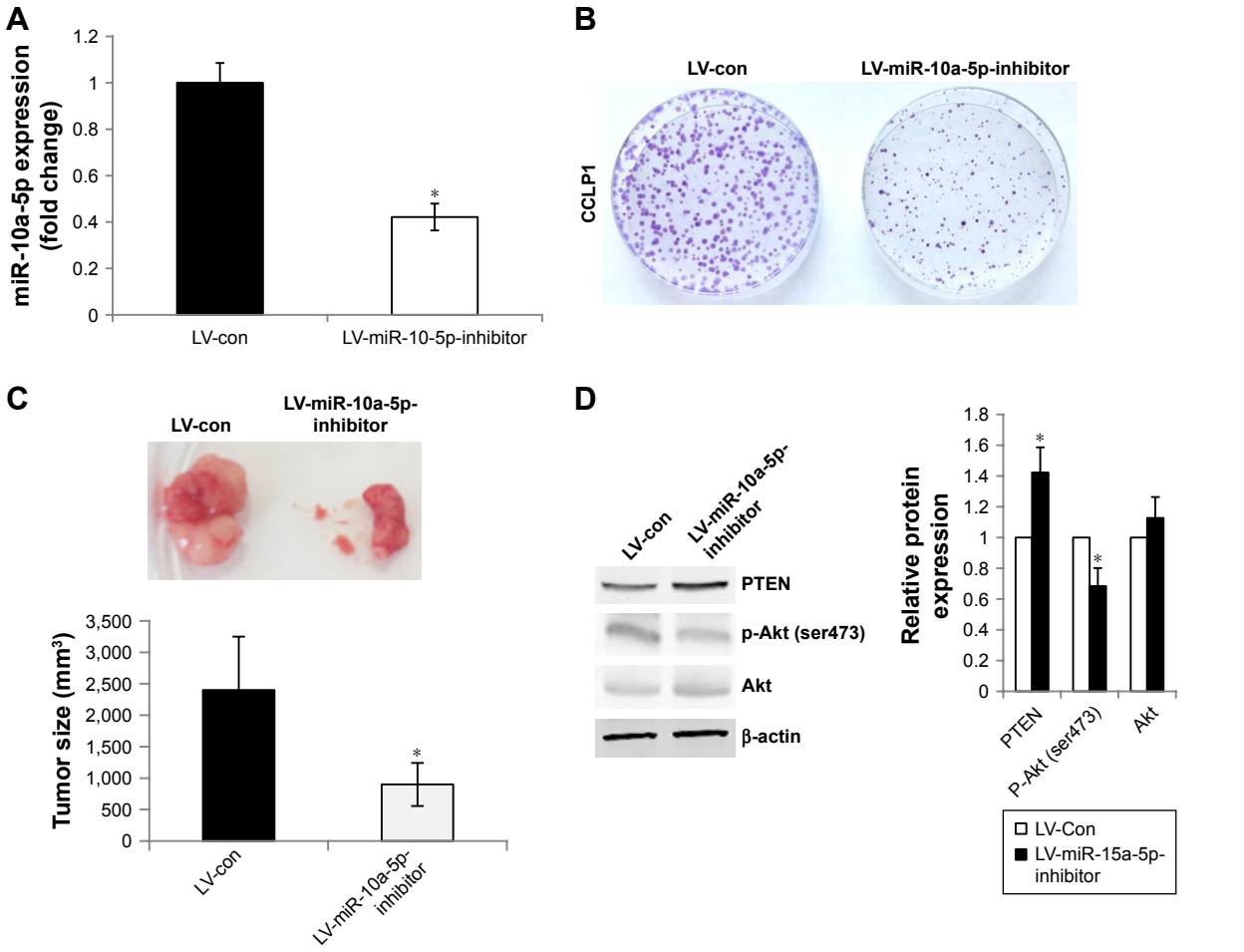

Figure 3 Inhibition of miR-10a-5p reduces tumor burden in vivo.
Notes: (**A**) miR-10a-5p expression was determined by qRT-PCR in CCLP1 cells with stable knockdown of miR-10a-5p (LV-miR-10a-5p-inhibitor) and control cells (LV-con). (**B**) Representative images of colony formation. (**C**) Representative image of tumors excised from LV-miR-10a-5p-inhibitor group and LV-con group (upper panel). Volume of xenograft tumors (lower panel). (**D**) Western blot analysis of PTEN, p-Akt (ser473), and total Akt in miR-10a-5p-inhibited and control xenograft tumor tissues. Quantifications of relative protein levels are shown at the right panel. Data were expressed as mean ± SD. *$P < 0.05$.

suggested that PTEN was a direct target of miR-10a-5p in CCA cells.

Inhibition of miR-10a-5p suppresses CCA growth in SCID mice

To further evaluate the effects of miR-10a-5p on CCA growth in vivo, we generated CCLP1 cells with stable knockdown of miR-10a-5p. CCLP1 cells were transfected with LV-mir-10a-5p-inhibitor or LV-con. As shown in Figure 3A, the downregulation of miR-10a-5p was confirmed by qRT-PCR. Knockdown of miR-10a-5p led to a significantly decreased colony formation in CCLP1 cells compared with control cells (Figure 3B). CCLP1 cells with stable knockdown of miR-10a-5p and control cells were injected subcutaneously into the flank of SCID mice to establish a xenograft model. Compared with the control group, knockdown of miR-10a-5p resulted in a significant reduction of tumor size and tumor volume (Figure 3C). Western blot analysis of the tumor tissues confirmed upregulated PTEN and decreased p-Akt (ser473) in miR-10a-5p knockdown tumors (Figure 3D). Taken together, these results suggested that inhibition of miR-10a-5p played an important role suppressed CCA cell proliferation.

Discussion

CCA is an aggressive tumor with very poor prognosis. The majority of patients present with unresectable disease and have a survival of less than 12 months following diagnosis.[35] It is crucial to understand the pathogenesis of CCA, find out the effective, targeted, individualized therapies, and improve the quality of patient's life. In our study, we investigated the effect of miR-10a-5p on CCA cells proliferation in vitro and in vivo. We found that overexpression of miR-10a-5p promoted CCA cells proliferation, whereas inhibition of miR-10a-5p suppressed proliferation and induced apoptosis in CCA cells. In a mouse xenograft model, inhibition of

miR-10a-5p significantly suppressed tumorigenicity. PTEN is a direct target of miR-10a-5p in CCA cells. Inhibition of miR-10a-5p led to the downregulation of Akt pathway. miRNA expression has been reported to be involved in tumor progression and prognosis, including CCA.[36] It has been reported that overexpression of miR-10a-5p promoted the migration and invasion of human HCC cell lines (QGY-7703 and HepG2) in vitro but suppressed metastasis in vivo.[37] EphA4 (Eph tyrosine kinase receptor) was identified as the direct target of miR-10a. miR-10a promotes HCC cell migration and invasion through targeting EphA4, thereby regulating epithelial–mesenchymal transition and cell adhesion.[37] Downregulation of miR-10a-5p has been shown to promote proliferation and restricts apoptosis via targeting T-box transcription factor 5 (TBX5) in inflamed synoviocytes.[38] In our study, we found that inhibition of miR-10a-5p suppressed CCA cells proliferation through regulating PTEN-Akt pathway.

Akt pathway has been well established as an important signaling intermediate controlling cell survival, growth, proliferation, and other cellular processes.[39] Activation of Akt pathway is an important survival pathway activated in cancer. Increased activation of AKT signaling was reproducibly observed in both CCA cell lines and CCA tissues.[40] PTEN is a tumor suppressor and is a major negative regulator of the Akt signaling pathway. PTEN can be regulated by posttranslational modifications that include oxidation, acetylation, phosphorylation, ubiquitination, and proteolytic cleavage and by protein–protein interactions.[41] PTEN can also be regulated by miRNAs. miRNAs may function as either oncogenes or tumor suppressors depending on their downstream targets.[42] For example, miR-21 contributes both HCC and CCA growth by targeting PTEN.[43,44] miR-221[45] and miR-17-92 cluster[46] promote CCA growth by targeting PTEN. In our study, we found that PTEN is a direct target of miR-10a-5p in CCA cells. Inhibition of miR-10a-5p promotes apoptosis in CCA cells through regulating PTEN.

Increasing evidences have shown that miRNAs are potential targets for human cancer treatment.[47] Our study provided insight into the mechanism of inhibition of miR-10a-5p suppressed CCA cells proliferation. miR-10a-5p may be serve as a potential target for CCA. These findings may help to better understand the tumorigenesis of CCA and develop effective therapeutic strategies.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Predicted targets of human miR-10a-5p

Target gene	Representative transcript	Gene name
BDNF	ENST00000439476.2	Brain-derived neurotrophic factor
ARSJ	ENST00000315366.7	Arylsulfatase family, member J
CRLF3	ENST00000324238.6	Cytokine receptor-like factor 3
HOXA3	ENST00000396352.4	Homeobox A3
VWC2L	ENST00000427124.1	von Willebrand factor C domain containing protein 2-like
RNF186	ENST00000375121.2	Ring finger protein 186
SOBP	ENST00000317357.5	Sine oculis binding protein homolog (*Drosophila*)
TFRC	ENST00000540528.1	Transferrin receptor
SMAP1	ENST00000370452.3	Small ArfGAP 1
KPNA5	ENST00000368564.1	Karyopherin alpha 5 (importin alpha 6)
HCN1	ENST00000303230.4	Hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
FIGN	ENST00000333129.3	Fidgetin
DAZAP1	ENST00000336761.6	DAZ-associated protein 1
HOXB3	ENST00000470495.1	Homeobox B3
NR6A1	ENST00000487099.2	Nuclear receptor subfamily 6, group A, member 1
KLHL29	ENST00000486442.1	Kelch-like family member 29
NCOR2	ENST00000405201.1	Nuclear receptor corepressor 2
ERGIC2	ENST00000360150.4	ERGIC and golgi 2
ELOVL2	ENST00000354666.3	ELOVL fatty acid elongase 2
USP46	ENST00000441222.3	Ubiquitin-specific peptidase 46
RPRD1A	ENST00000399022.4	Regulation of nuclear pre-mRNA domain containing 1A
FLJ20373	ENST00000414004.2	
SDC1	ENST00000254351.4	Syndecan 1
KCNA6	ENST00000433855.1	Potassium voltage-gated channel, shaker-related subfamily, member 6
CADM2	ENST00000383699.3	Cell adhesion molecule 2
FLRT2	ENST00000330753.4	Fibronectin leucine-rich transmembrane protein 2
LIX1L	ENST00000369308.3	Lix1 homolog (mouse)-like
RORB	ENST00000376896.3	RAR-related orphan receptor B
RORA	ENST00000335670.6	RAR-related orphan receptor A
CYTH1	ENST00000585509.1	Cytohesin 1
SMTNL2	ENST00000338859.4	Smoothelin-like 2
GOLGA3	ENST00000204726.3	Golgin A3
ATCAY	ENST00000450849.2	Ataxia, cerebellar, Cayman type
MAP3K7	ENST00000369325.3	Mitogen-activated protein kinase kinase kinase 7
UBE2I	ENST00000355803.4	Ubiquitin-conjugating enzyme E2I
TMEM183A	ENST00000367242.3	Transmembrane protein 183A
ERI3	ENST00000372259.5	ERI1 exoribonuclease family member 3
ATXN2	ENST00000550104.1	Ataxin 2
XRN1	ENST00000264951.4	5′-3′ exoribonuclease 1
LRRC8B	ENST00000330947.2	Leucine-rich repeat containing 8 family, member B
GABRB2	ENST00000393959.1	Gamma-aminobutyric acid (GABA) A receptor, beta 2
CNNM4	ENST00000540067.1	Cyclin M4
IL1RAPL1	ENST00000378993.1	Interleukin 1 receptor accessory protein-like 1
ZMYND11	ENST00000381591.1	Zinc finger, MYND-type containing 11
IGDCC4	ENST00000352385.2	Immunoglobulin superfamily, DCC subclass, member 4
ALPL	ENST00000374840.3	Alkaline phosphatase, liver/bone/kidney
KLF7	ENST00000423015.1	Kruppel-like factor 7 (ubiquitous)
NPAS3	ENST00000346562.2	Neuronal PAS domain protein 3
CECR6	ENST00000399875.1	Cat eye syndrome chromosome region, candidate 6
SSX2IP	ENST00000342203.3	Synovial sarcoma, X breakpoint 2 interacting protein
ZNF367	ENST00000375256.4	Zinc finger protein 367
E2F7	ENST00000416496.2	E2F transcription factor 7
CELF2	ENST00000379261.4	CUGBP, Elav-like family member 2
SNX18	ENST00000343017.6	Sorting nexin 18
ONECUT1	ENST00000560699.2	One cut homeobox 1

(*Continued*)

Table S1 (*Continued*)

Target gene	Representative transcript	Gene name
CTD-2267D19.3	ENST00000578774.1	Uncharacterized protein
PRKAA2	ENST00000371244.4	Protein kinase, AMP-activated, alpha 2 catalytic subunit
ELOVL6	ENST00000394607.3	ELOVL fatty acid elongase 6
H3F3C	ENST00000340398.3	H3 histone, family 3C
H3F3B	ENST00000591890.1	H3 histone, family 3B (H3.3B)
ESRRG	ENST00000361525.3	Estrogen-related receptor gamma
BAZ1B	ENST00000339594.4	Bromodomain adjacent to zinc finger domain, 1B
FNBP1L	ENST00000370253.2	Formin binding protein 1-like
PAPD5	ENST00000357464.3	PAP-associated domain containing 5
TBX5	ENST00000349716.5	T-box 5
CSRNP3	ENST00000314499.7	Cysteine-serine-rich nuclear protein 3
BBX	ENST00000415149.2	Bobby sox homolog (*Drosophila*)
FAM196A	ENST00000522781.1	Family with sequence similarity 196, member A
PRRT3	ENST00000412055.1	Proline-rich transmembrane protein 3
IGSF1	ENST00000370900.1	Immunoglobulin superfamily, member 1
ACTG1	ENST00000331925.2	Actin, gamma 1
EPHA2	ENST00000358432.5	EPH receptor A2
KIAA0247	ENST00000342745.4	KIAA0247
MDGA2	ENST00000439988.3	MAM domain-containing glycosylphosphatidylinositol anchor protein 2
HNRNPK	ENST00000376281.4	Heterogeneous nuclear ribonucleoprotein K
JARID2	ENST00000341776.2	Jumonji, AT-rich interactive domain 2
KCTD16	ENST00000507359.3	Potassium channel tetramerization domain containing 16
PALM2	ENST00000448454.2	Paralemmin 2
WWC2	ENST00000403733.3	WW and C2 domain containing 2
NR4A3	ENST00000330847.1	Nuclear receptor subfamily 4, group A, member 3
NEDD4	ENST00000338963.2	Neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase
BCL6	ENST00000406870.2	B-cell CLL/lymphoma 6
RP6-24A23.6	ENST00000563887.1	Uncharacterized protein
CTNNBIP1	ENST00000377263.1	Catenin, beta-interacting protein 1
WBP11	ENST00000261167.2	WW domain binding protein 11
TRIM2	ENST00000338700.5	Tripartite motif containing 2
ZFAND5	ENST00000237937.3	Zinc finger, AN1-type domain 5
ANXA7	ENST00000372921.5	Annexin A7
CAMK2B	ENST00000457475.1	Calcium/calmodulin-dependent protein kinase II beta
MTMR3	ENST00000333027.3	Myotubularin-related protein 3
CTDSPL	ENST00000443503.2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like
EPHA5	ENST00000273854.3	EPH receptor A5
SVOP	ENST00000299134.5	SV2-related protein homolog (rat)
ST6GALNAC6	ENST00000373146.1	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6
RYBP	ENST00000477973.2	RING1 and YY1 binding protein
ELAVL2	ENST00000380110.4	ELAV like neuron-specific RNA-binding protein 2
KIAA1429	ENST00000437199.1	KIAA1429
NR2C2	ENST00000425241.1	Nuclear receptor subfamily 2, group C, member 2
TMEM167B	ENST00000338272.8	Transmembrane protein 167B
KLHDC10	ENST00000335420.5	Kelch domain containing 10
GATA3	ENST00000379328.3	GATA-binding protein 3
PRR15L	ENST00000300557.2	Proline-rich 15-like
SH3D19	ENST00000409598.4	SH3 domain containing 19
ITSN1	ENST00000379960.5	Intersectin 1 (SH3 domain protein)
CLASP2	ENST00000539981.1	Cytoplasmic linker-associated protein 2
FXR2	ENST00000250113.7	Fragile X mental retardation, autosomal homolog 2
ANKFY1	ENST00000341657.4	Ankyrin repeat and FYVE domain containing 1
E2F3	ENST00000346618.3	E2F transcription factor 3
SNX12	ENST00000374274.3	Sorting nexin 12

(*Continued*)

Table S1 (*Continued*)

Target gene	Representative transcript	Gene name
MTF2	ENST00000370298.4	Metal response element binding transcription factor 2
SERTAD4	ENST00000367012.3	SERTA domain containing 4
TMEM168	ENST00000312814.6	Transmembrane protein 168
SHANK3	ENST00000414786.2	SH3 and multiple ankyrin repeat domains 3
ZNF280C	ENST00000370978.4	Zinc finger protein 280C
HOXA1	ENST00000355633.5	Homeobox A1
PDE7A	ENST00000401827.3	Phosphodiesterase 7A
DPF2	ENST00000528416.1	D4, zinc and double PHD fingers family 2
CDK6	ENST00000265734.4	Cyclin-dependent kinase 6
CRK	ENST00000398970.5	v-crk avian sarcoma virus CT10 oncogene homolog
EBF2	ENST00000535548.1	Early B-cell factor 2
LPHN1	ENST00000340736.6	Latrophilin 1
TBC1D22B	ENST00000373491.3	TBC1 domain family, member 22B
NFIX	ENST00000360105.4	Nuclear factor I/X (CCAAT-binding transcription factor)
BLZF1	ENST00000367808.3	Basic leucine zipper nuclear factor 1
CBX5	ENST00000209875.4	Chromobox homolog 5
CCNK	ENST00000389879.5	Cyclin K
PDE12	ENST00000311180.8	Phosphodiesterase 12
FAM76A	ENST00000373954.6	Family with sequence similarity 76, member A
BMP2K	ENST00000335016.5	BMP2 inducible kinase
GPCPD1	ENST00000379019.4	Glycerophosphocholine phosphodiesterase GDE1 homolog (*S. cerevisiae*)
MTF1	ENST00000373036.4	Metal-regulatory transcription factor 1
MAP3K13	ENST00000448876.1	Mitogen-activated protein kinase kinase kinase 13
ANK1	ENST00000289734.7	Ankyrin 1, erythrocytic
PTEN	ENST00000371953.3	Phosphatase and tensin homolog
MANEAL	ENST00000397631.3	Mannosidase, endo-alpha-like
LANCL1	ENST00000443314.1	LanC lantibiotic synthetase component C-like 1 (bacterial)
SLC6A5	ENST00000525748.1	Solute carrier family 6 (neurotransmitter transporter), member 5
ARIH2	ENST00000356401.4	Ariadne RBR E3 ubiquitin protein ligase 2
FOSL2	ENST00000379619.1	FOS-like antigen 2
NR5A2	ENST00000367362.3	Nuclear receptor subfamily 5, group A, member 2
TRIM66	ENST00000299550.6	Tripartite motif containing 66
GPR61	ENST00000527748.1	G protein-coupled receptor 61
KLC2	ENST00000394065.2	Kinesin light chain 2
MAPKBP1	ENST00000457542.2	Mitogen-activated protein kinase binding protein 1
BAZ2B	ENST00000392782.1	Bromodomain adjacent to zinc finger domain, 2B
FBXO30	ENST00000237281.4	F-box protein 30
SLC38A2	ENST00000256689.5	Solute carrier family 38, member 2
NUP50	ENST00000347635.4	Nucleoporin 50 kDa
PEA15	ENST00000360472.4	Phosphoprotein enriched in astrocytes 15
TSPAN9	ENST00000537971.1	Tetraspanin 9
CREB1	ENST00000432329.2	cAMP responsive element binding protein 1
GCLM	ENST00000370238.3	Glutamate-cysteine ligase, modifier subunit
AAK1	ENST00000409085.4	AP2 associated kinase 1
ARRDC3	ENST00000265138.3	Arrestin domain containing 3
SRSF1	ENST00000258962.4	Serine/arginine-rich splicing factor 1
CNOT4	ENST00000541284.1	CCR4-NOT transcription complex, subunit 4
MTSS1L	ENST00000338779.6	Metastasis suppressor 1-like
PDE4A	ENST00000380702.2	Phosphodiesterase 4A, cAMP-specific
PEX5L	ENST00000467460.1	Peroxisomal biogenesis factor 5-like
IFFO2	ENST00000455833.2	Intermediate filament family orphan 2
KIAA1462	ENST00000375377.1	KIAA1462
NFE2L1	ENST00000585291.1	Nuclear factor, erythroid 2-like 1
MYT1L	ENST00000399161.2	Myelin transcription factor 1-like
MIEF1	ENST00000325301.2	Mitochondrial elongation factor 1
NCOA6	ENST00000374796.2	Nuclear receptor coactivator 6
RNF180	ENST00000389100.4	Ring finger protein 180

(*Continued*)

Table S1 (*Continued*)

Target gene	Representative transcript	Gene name
FRS2	ENST00000550389.1	Fibroblast growth factor receptor substrate 2
RASAL2	ENST00000448150.3	RAS protein activator like 2
TENM2	ENST00000519204.1	Teneurin transmembrane protein 2
ZNF608	ENST00000504926.1	Zinc finger protein 608
FZD2	ENST00000315323.3	Frizzled family receptor 2
ARHGEF12	ENST00000397843.2	Rho guanine nucleotide exchange factor (GEF) 12
MYCBP	ENST00000397572.2	MYC binding protein
BACH2	ENST00000257749.4	BTB and CNC homology 1, basic leucine zipper transcription factor 2
MLLT6	ENST00000325718.7	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 6
TBL1X	ENST00000407597.2	Transducin (beta)-like 1X-linked
ATF2	ENST00000487334.2	Activating transcription factor 2
GINS2	ENST00000253462.3	GINS complex subunit 2 (Psf2 homolog)
FLT1	ENST00000282397.4	fms-related tyrosine kinase 1
CEP85L	ENST00000368491.3	Centrosomal protein 85 kDa-like
BEND3	ENST00000369042.1	BEN domain containing 3
SPAG9	ENST00000262013.7	Sperm-associated antigen 9
KCTD17	ENST00000402077.3	Potassium channel tetramerization domain containing 17
USF2	ENST00000594064.1	Upstream transcription factor 2, c-fos interacting
LGALSL	ENST00000409537.2	Lectin, galactoside-binding-like
TPP2	ENST00000376052.3	Tripeptidyl peptidase II
DLGAP2	ENST00000421627.2	Discs, large (*Drosophila*) homolog-associated protein 2
TMEM170B	ENST00000379426.1	Transmembrane protein 170B
ZBTB43	ENST00000449886.1	Zinc finger and BTB domain containing 43
L3MBTL3	ENST00000529410.1	l(3)mbt-like 3 (*Drosophila*)
KIAA1549	ENST00000440172.1	KIAA1549
TNRC6B	ENST00000335727.9	Trinucleotide repeat containing 6B
SAMD14	ENST00000330175.4	Sterile alpha motif domain containing 14
INO80D	ENST00000403263.1	INO80 complex subunit D
CALCR	ENST00000359558.2	Calcitonin receptor
TGOLN2	ENST00000377386.3	Trans-golgi network protein 2
TET2	ENST00000545826.1	tet methylcytosine dioxygenase 2
SUFU	ENST00000369902.3	Suppressor of fused homolog (*Drosophila*)
FADS3	ENST00000540820.1	Fatty acid desaturase 3
LEPRE1	ENST00000236040.4	Leucine proline-enriched proteoglycan (leprecan) 1
CAMK2G	ENST00000351293.3	Calcium/calmodulin-dependent protein kinase II gamma
NFAT5	ENST00000354436.2	Nuclear factor of activated T-cells 5, tonicity-responsive
MED1	ENST00000300651.6	Mediator complex subunit 1
CNOT6	ENST00000393356.1	CCR4-NOT transcription complex, subunit 6
RP11-766F14.2	ENST00000511828.1	Protein LOC285556
STARD13	ENST00000336934.5	StAR-related lipid transfer (START) domain containing 13
LCOR	ENST00000371103.3	Ligand-dependent nuclear receptor corepressor
HDAC4	ENST00000345617.3	Histone deacetylase 4
CCDC71L	ENST00000523505.1	Coiled-coil domain containing 71-like
ST8SIA1	ENST00000396037.4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
MBD5	ENST00000407073.1	Methyl-CpG binding domain protein 5
RBMS3	ENST00000396583.3	RNA binding motif, single stranded interacting protein 3
THRA	ENST00000450525.2	Thyroid hormone receptor, alpha
BCL2L11	ENST00000393256.3	BCL2-like 11 (apoptosis facilitator)
FBXO28	ENST00000424254.2	F-box protein 28
NT5DC1	ENST00000319550.4	5′-nucleotidase domain containing 1
POLR3H	ENST00000396504.2	Polymerase (RNA) III (DNA directed) polypeptide H (22.9 kD)
ANK3	ENST00000280772.2	Ankyrin 3, node of Ranvier (ankyrin G)
BICD2	ENST00000356884.6	Bicaudal D homolog 2 (*Drosophila*)
PAPOLA	ENST00000557471.1	Poly(A) polymerase alpha
ZXDC	ENST00000389709.3	ZXD family zinc finger C
DLG5	ENST00000372391.2	Discs, large homolog 5 (*Drosophila*)

(*Continued*)

Table S1 (*Continued*)

Target gene	Representative transcript	Gene name
CCDC88A	ENST00000336838.6	Coiled-coil domain containing 88A
LYVE1	ENST00000256178.3	Lymphatic vessel endothelial hyaluronan receptor 1
PURB	ENST00000395699.2	Purine-rich element binding protein B
GJA9	ENST00000454994.2	Gap junction protein, alpha 9, 59 kDa
KCNH5	ENST00000322893.7	Potassium voltage-gated channel, subfamily H (eag-related), member 5
WNK3	ENST00000375169.3	WNK lysine deficient protein kinase 3
STRN	ENST00000263918.4	Striatin, calmodulin binding protein
UNC5B	ENST00000335350.6	unc-5 homolog B (*C. elegans*)
FKBP15	ENST00000238256.3	FK506 binding protein 15, 133 kDa
SHISA7	ENST00000376325.4	Shisa family member 7
AGO3	ENST00000373191.4	Argonaute RISC catalytic component 3
CELF6	ENST00000287202.5	CUGBP, Elav-like family member 6
MAP3K2	ENST00000409947.1	Mitogen-activated protein kinase kinase kinase 2
TIAM1	ENST00000286827.3	T-cell lymphoma invasion and metastasis 1
SCN3A	ENST00000360093.3	Sodium channel, voltage-gated, type III, alpha subunit
ZDHHC18	ENST00000374142.4	Zinc finger, DHHC-type containing 18
ONECUT2	ENST00000491143.2	One cut homeobox 2
SPTY2D1	ENST00000336349.5	SPT2, Suppressor of Ty, domain containing 1 (*S. cerevisiae*)
CHD6	ENST00000373233.3	Chromodomain helicase DNA binding protein 6
AKAP2	ENST00000374525.1	A kinase (PRKA) anchor protein 2
BTRC	ENST00000370187.3	Beta-transducin repeat containing E3 ubiquitin protein ligase
SMURF1	ENST00000361368.2	SMAD-specific E3 ubiquitin protein ligase 1
EPHA4	ENST00000281821.2	EPH receptor A4
WDR26	ENST00000414423.2	WD repeat domain 26
GATAD2A	ENST00000360315.3	GATA zinc finger domain containing 2A
RIMS2	ENST00000507740.1	Regulating synaptic membrane exocytosis 2
PURG	ENST00000475541.1	Purine-rich element binding protein G
PALM2-AKAP2	ENST00000374530.3	PALM2-AKAP2 read through
NFASC	ENST00000401399.1	Neurofascin
ELAVL3	ENST00000359227.3	ELAV like neuron-specific RNA binding protein 3
LHFPL4	ENST00000287585.6	Lipoma HMGIC fusion partner-like 4
ARNT	ENST00000358595.5	Aryl hydrocarbon receptor nuclear translocator
STK35	ENST00000381482.3	Serine/threonine kinase 35
CEP350	ENST00000367607.3	Centrosomal protein 350 kDa
ZBTB16	ENST00000335953.4	Zinc finger and BTB domain containing 16
NUFIP2	ENST00000225388.4	Nuclear fragile X mental retardation protein interacting protein 2
CLCN5	ENST00000376088.3	Chloride channel, voltage-sensitive 5
C3orf14	ENST00000494481.1	Chromosome 3 open reading frame 14
TFAP2A	ENST00000379613.3	Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)
CSMD1	ENST00000400186.3	CUB and Sushi multiple domains 1
MAPRE1	ENST00000375571.5	Microtubule-associated protein, RP/EB family, member 1
UBXN7	ENST00000296328.4	UBX domain protein 7
WAPAL	ENST00000298767.5	Wings apart-like homolog (*Drosophila*)
SLC9A7	ENST00000328306.4	Solute carrier family 9, subfamily A (NHE7, cation proton antiporter 7), member 7
ZDHHC21	ENST00000380916.4	Zinc finger, DHHC-type containing 21
RB1CC1	ENST00000025008.5	RB1-inducible coiled-coil 1
DVL3	ENST00000313143.3	Dishevelled segment polarity protein 3
SMAD2	ENST00000262160.6	SMAD family member 2
MCMBP	ENST00000360003.3	Minichromosome maintenance complex binding protein
OTUD7A	ENST00000307050.4	OTU domain containing 7A
AFF4	ENST00000265343.5	AF4/FMR2 family, member 4
KCNC3	ENST00000376959.2	Potassium voltage-gated channel, Shaw-related subfamily, member 3
SLC25A1	ENST00000215882.5	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1
C14orf28	ENST00000325192.3	Chromosome 14 open reading frame 28
SESN3	ENST00000536441.1	Sestrin 3
SCARB2	ENST00000264896.2	Scavenger receptor class B, member 2
ZNF202	ENST00000336139.4	Zinc finger protein 202

(*Continued*)

Table S1 (*Continued*)

Target gene	Representative transcript	Gene name
SLC35G1	ENST00000371408.3	Solute carrier family 35, member G1
IRS1	ENST00000305123.5	Insulin receptor substrate 1
AHSA2	ENST00000394457.3	AHA1, activator of heat shock 90 kDa protein ATPase homolog 2 (yeast)
CADM1	ENST00000452722.3	Cell adhesion molecule 1
HTT	ENST00000355072.5	Huntingtin
CNTNAP5	ENST00000431078.1	Contactin associated protein-like 5
ZNF827	ENST00000379448.4	Zinc finger protein 827
CDH19	ENST00000540086.1	Cadherin 19, type 2