An Artificially Designed Interfering IncRNA Expressed by Oncolytic Adenovirus Competitively Consumes OncomiRs to Exert Antitumor Efficacy in Hepatocellular Carcinoma

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Abstract

Endogenous miRNAs, especially oncogenic miRNAs (OncomiR), have been molecular targets for cancer therapy. We generated an artificially designed interfering long non-coding RNA (lncRNAi), which contains the sequences that can complementarily bind to multiple OncomiRs and is expressed by cancer-selectively replicating adenovirus. The adenovirus-expressed lncRNAi with high levels in hepatocellular carcinoma (HCC) cells competes with OncomiR target genes to bind to and consume OncomiRs, thereby achieving the targeted anti-HCC efficacy. With the targeting replication of adenovirus in HCC cells, lncRNAi was highly expressed and resulted in decreased abilities of proliferation, migration, and invasion, induced cell-cycle changes and apoptosis, and markedly changed the cellular miRNA and miRNA expression profiles in HCC cells. The optimal antitumor effect was also demonstrated on HCC cell line xenograft models and HCC patient-derived xenograft (PDX) tumor models in nude mice. This strategy has established a technology platform with a reliable therapeutic effect for HCC therapy.

Introduction

Bioinformatics analysis has shown that a significant number of human genes are regulated by miRNAs, which are small molecules that are large in number, widely distributed, and highly effective, indicating that miRNAs are important components of gene regulation network (1, 2). Studies have shown that the highly expressed miRNAs in cancer cells are involved in origin, proliferation, invasion, and metastasis of tumor cells (3), which are considered a group of oncogenic miRNAs (OncomiR). The expression of some miRNAs in tumor cells is downregulated or even lost, resulting in the induction or promotion of tumor progression. This group of miRNAs can be considered the tumor suppressor miRNAs. miRNAs form an RNA-induced silencing complex (RISC) with other proteins and bind to the mRNAs of target genes through complementary nucleic acid sequences, resulting in degradation of target gene mRNA or inhibition of its translation, which ultimately achieves posttranscriptional regulation of target genes (4). The highly expressed OncomiRs in tumor cells can inactivate some tumor suppressor genes. In contrast, the expression of some tumor suppressor miRNAs is downregulated or inactivated, rendering them unable to regulate or monitor function of corresponding oncogenes, thereby allowing these oncogenes to increasingly express. Therefore, the high expression of OncomiRs and the low expression of tumor suppressor miRNAs are important factors that promote tumorigenesis. Interference of miRNA expression profile in tumor cells has significant impacts on the biologic characteristics of tumor cells (5, 6). Therefore, the value of miRNAs in targeted cancer therapy is inestimable.

The difference in the miRNA expression profiles between tumor cells and normal tissue cells, as well as the specificity of the miRNA expression profiles between different tumors and the roles of miRNAs in cancer development, have provided useful molecular targets for the treatment of tumors. Recently, there have been many therapeutic strategies for tumors targeting miRNAs (7). With regard to overexpressed OncomiRs in tumor cells, studies have used miRNA inhibitors or antisense sequences to block miRNA expression, thereby inhibiting tumor growth (8, 9). However, most of the existing therapeutic studies focus on single miRNA or its family. Because the regulatory mechanisms of miRNAs are complex, one miRNA can regulate multiple target genes, and one target gene can be regulated by multiple miRNAs. Tumorigenesis and its progression involve many miRNAs, which regulate the expression of even more target genes or affect many signal transduction pathways. Therefore, the interference of single miRNA expression has a limited inhibitory effect on tumors, while...
cancer cells can easily regain their proliferation activity through bypassing the signaling pathway. Joint interference of multiple miRNAs with different or complementary mechanisms would inhibit broad signal transduction pathways, which would be more effective for tumor treatment.

Long noncoding RNA (lncRNA) transcripts are produced in various cells and have been functionally associated with human cancer. The lncRNAs have multiple functions, such as chromatin remodeling, chromatin interactions, competing endogenous RNAs (ceRNA), and natural antisense transcripts (10). On the basis of the working mechanisms of miRNAs and the characteristics of IncRNAs, we generated an artificially designed interfering long noncoding RNA (lncRNAi) which was introduced into tumor cells by adenovirus. This lncRNAi simultaneously contains sequences that can complementarily bind to multiple OncomiRs. Therefore, it can compete with the miRNAs of OncomiR target genes to bind to OncomiRs, which exhausts the high levels of OncomiRs, thereby achieving the interference of multiple miRNAs and protecting the tumor suppressor genes that are the targets of OncomiRs. However, such competitive protection effect requires that the copy number of the competitor (i.e., lncRNAi) be significantly higher than the target being competed (i.e., mRNA of OncomiR target gene) to obtain a better effect. Higher ratio of the copy number of lncRNAi versus miRNAs results in a lower binding efficiency of OncomiRs to target gene miRNAs and a maximal protection of target gene expression. Up to now, there are no studies that used IncRNAi for the treatment of cancers.

The miRNAs that are highly expressed in hepatocellular carcinoma (HCC) include miR21 (11), miR221/222 (11, 12), miR224 (13), miR17-5p/20a (14), miR10b (15), miR106b (16), miR151-5p (17), miR155 (18), miR181a/181b (19), miR184 (20), etc. These miRNAs play different roles in the tumorigenesis and progression of HCC (21, 22). Of the OncomiRs that were reported to highly express in HCC, we selected the 12 OncomiRs, including miR21, miR221/222, miR224, miR17-5p/20a, miR10b, miR106b, miR151-5p, miR155, miR181a/181b, miR184, miR1, and miR501-5p. These miRNAs have a complementary effects and act on different target genes and signal transduction pathways during the tumorigenesis and progression of HCC, and also play regulatory roles in the proliferation, invasion, metastasis, and apoptosis of HCC cells. The current study provides an anticancer strategy for the treatment of HCC by competitively binding OncomiRs. This strategy uses an oncolytic adenoviral vector, which we constructed previously and were confirmed experimentally to be able to specifically replicate in high copy number in HCC cells, to express an artificially designed IncRNAi containing the complementary binding sequences to the seed sequences of the above-mentioned 12 OncomiRs. With the cancer-selective replication of the adenovirus, IncRNAi is highly expressed and accumulated in high copy number in cancer cells. Through the competition with the target gene miRNAs of OncomiRs to bind to OncomiRs and consume OncomiRs, IncRNAi protects a large number of tumor suppressor genes from being inhibited by OncomiRs, thus achieving the targeted intervention treatment of cancer cells. Because of the tumor-targeted replication of the virus and the specific interference of the multiple OncomiRs, such a strategy allows the anticancer efficacy to be increased and the safety to be improved. Thus, this strategy has a good prospect for clinical application.

Materials and Methods

Construction of the viral vectors

The Survivin promoter-regulated cancer-selectively replicating adenovirus AdSurp (23–25), which we previously recombined and named oncolytic adenovirus because of its capability to lyse tumor cells, was used as the vector to express the artificially designed IncRNAi to allow a targeted transcription and expression of IncRNAi in high copy number in HCC cells. The complementary sequences of 12 OncomiR seed sequences (Supplementary Table S1) were connected in tandem and repeated six times as the encoding gene for IncRNAi. A stop codon (TAG) was introduced into the beginning of IncRNAi coding sequence and the 3′ end, respectively. A poly (A) tail sequence was added at the 3′ end. The IncRNAi gene was inserted into the AdSurp genome downstream of the E1a gene open reading frame. A nonreplicative control adenovirus, AdSVpEGFP-InR, which carried the IncRNAi but used the enhanced GFP (eGFP) reporter gene to substitute the E1a, was simultaneously constructed. The previously constructed Ad5-eGFP was used as a negative control virus. All viruses were amplified in HEK293 cells and purified using the cesium chloride gradient centrifugation method. The titers of viruses were determined by the TCID50 method.

 Luciferase assay

The sequence (5′-TCTGATAAGCTAGTAAGCACTTTA-3′), which was recognized to bind by OncomiRs miR21 and miR17-5p, was inserted into the 3′-untranslated region (UTR) of the luciferase vector pGL3-Control (Promega Corporation) to generate the plasmid pGL3-miR21/17-5p. The luciferase assay was performed in HepG2 and L02 cells after transfected with the OncomiR inhibitors (GenePharma Inc.) and infected with AdSVPE1a-InR. The relative luciferase activity of pGL3-miR21/17-5p was normalized with the data of positive control pGL3-Control.

Culture of cell lines and detection of gene expression

HCC cell lines (HepG2, Hep3B, SMCC-7721, MHCC97H, MHCC97L, Huh-7, and PLC/PRF/5) and normal liver cell lines (L02 and WRL-68) were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) on November 2015, which were authenticated and tested by the short tandem repeat (STR) method. The cells were cultured in 6-well plates at 1 × 10^6 cells/well following the manufacturer's instructions. On the basis of these experiments, cells were infected with different titers of the experimental endogenous and control adenoviruses to establish the virus-infected sub-cell lines. HepG2 cells were transfected with inhibitors of miR21 and miR17-5p at a concentration of 10 μg/well. After the parental cells and the cell sublines were harvested, total cell lysates were prepared, and the expression of proteins was examined by Western blot analysis with the rabbit-anti-E1a antibody (Cell Signaling Technology, Inc.) and mouse anti-Survivin antibody (Abcam). All other antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology, Inc.

Detection of adenovirus selectively replicating ability

The abovementioned HCC cells and normal liver cells were harvested and seeded in 96-well plates (1 × 10^5 cells/well). After cell attachment, the medium was changed to serum-free medium. The AdSVPE1a-InR virus was added at a multiplicity of infection.
(MOI) of 1 pfu/cell, and the AdSVPeGFP-lncR virus was used as a control. Two hours after virus infection, cells were refed with medium containing 5% serum and cultured for 0, 24, 48, and 72 hours. Cells were harvested, and the viral titer was detected with the TCID50 method. Cells infected with the AdSVPeGFP-lncR control virus were observed under a fluorescent microscope to examine eGFP expression.

PCR detection of adenovirus-mediated lncRNAi expression
The abovementioned cell lines were infected with the AdSVPE1a-lncR virus (MOI = 1 pfu/cell), and the AdSVPeGFP-lncR was used as a control. After culture for 48 hours, cells were harvested and total RNA was extracted. Conventional RT-PCR and quantitative real-time PCR (qRT-PCR) were used to detect lncRNAi expression. The following lncRNAi-specific PCR primers were used: LncR-F, 5'-CTGCAGCTGGCTACAGCTTTA-3', LncR-R, 5'-ACATTACATTGCTGTCCGGTTG-3'.

Detection of cell proliferative ability
The effect of virus AdSVPE1a-lncR on the proliferation of HCC cells and normal cells was detected by the tetrazolium colorimetric assay (MTT assay), and AdSVPeGFP-lncR and Ad5-eGFP were used as controls. Cells were diluted with 10% serum-containing culture medium and seeded in 96-well plates (1 x 10^4 cells/well in 100 μL). After cell attachment, virus was diluted with serum-free culture medium, and 100 μL of corresponding virus was added (MOI = 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, and 500 pfu/cell). For each MOI value, eight replicate wells were prepared. The cells were incubated at 37°C for 2 hours, and the cell culture medium was then changed to serum-containing medium (100 μL/well). After culture for 48 hours, the medium was discarded and 100 μL of 0.1 mol/L PBS was added to each well. The MTT labeling agent (10 μL/well) was then added at a final concentration of 0.5 mg/mL, and the cells were incubated in an incubator for 4 hours. Solubilization solution (10% SDS in 0.01 mol/L HCl) was then added (100 μL/well), and the plate was incubated overnight in an incubator. The absorbance at 490 nm was determined by a plate reader, and the cell survival curves were plotted.

Detection of cell migration and invasion ability
The abovementioned cell lines were cultured and infected with AdSVPE1a-lncR (MOI = 1 pfu/cell). The AdSVPeGFP-lncR and Ad5-eGFP were used as control groups, the parental cells were cultured at the same time as a blank control. Cells were harvested 48 hours after virus infection. A portion of the cells was fixed with prechilled 75% ethanol and incubated at 4°C overnight. Cells were washed twice with PBS, and RNase-containing propidium iodide (PI) solution was added to stain cells for 30 minutes in darkness. FCM was used to determine the cell-cycle status. The remaining cells were stained with Annexin V/PI and apoptosis was determined by flow cytometry (FCM).

Detection of changes in cellular gene expression profile
The Huh-7, HepG2, SMMC-7721, Hep3B, and L02 cells were seeded in a 24-well plate (1 x 10^4 cells/well in 100 μL). After cell attachment, the virus was diluted with serum-free medium, 100 μL of AdSVPE1a-lncR or AdSVPeGFP-lncR was added (MOI = 1 pfu/cell), and the parental cells were synchronously cultured as a blank control. Cells were cultured in an incubator for 2 hours and then refed with 100 μL/well of serum-containing medium. After culture for 48 hours, cells were harvested for gene expression analysis using Agilent Whole Human Genome (4 x 44K) Microarray (Agilent Technologies) in the National Engineering Center for Biochip, Shanghai Biotechnology Corporation (Shanghai, China). Total RNA was extracted and purified using the miVana miRNA Isolation Kit (Ambion) following the manufacturer's instructions and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent Technologies). The prepared RNA samples were amplified and labeled by the Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies). Labeled cRNA was purified by the RNeasy Mini Kit (QIAGEN). Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using the Gene Expression Hybridization Kit (Agilent Technologies) in hybridization oven. After hybridization for 17 hours, slides were washed in staining dishes (Thermo Shandon) with the Gene Expression Wash Buffer Kit (Agilent Technologies), and scanned by the Agilent Microarray Scanner (Agilent Technologies). Data were extracted with the Feature Extraction Software 10.7 (Agilent Technologies). Raw data were normalized by the Quantile algorithm, GeneSpring Software 12.6.1 (Agilent Technologies). A part of harvested HepG2 cells was prepared for examining the expression of OncomirR-presentative target proteins, such as PTEN, p27kip1, CDK4, p38/MAPK, and Survivin by Western blot analysis.

Detection of changes in cellular miRNA expression profile
The Huh-7, HepG2, SMMC-7721, Hep3B, and L02 cells were infected with the AdSVPE1a-lncR and AdSVPeGFP-lncR as aforementioned. After 48 hours, cells were harvested for miRNA expression analysis using the Agilent Human miRNA (8 x 60K) Microarray (Agilent Technologies). The procedures for RNA extraction and purification were performed following the same steps with the Agilent Whole Human Genome Microarray. Molecular miRNA in total RNA was labeled and each slide was hybridized with 100 ng Cy3-labeled RNA in hybridization oven using the miRNA Complete Labeling and Hybridization Kit (Agilent Technologies). After hybridization, slides were washed in staining dishes with the Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned by the Agilent Microarray Scanner (Agilent Technologies) and the Feature Extraction Software 10.7 (Agilent Technologies). Raw data were normalized by the Quantile algorithm, Gene Spring Software 12.6 (Agilent Technologies). The
expression of 12 example OncomiRs in HCC cells after transfection of lncRNAi was also detected by qRT-PCR.

HCC cell line xenograft models in nude mice

The experiments of HCC cell line xenograft mouse models were approved by the Animal Ethics Committee of Second Military Medical University (Shanghai, China); all experiments were performed in accordance with the relevant guidelines and regulations. Sixty healthy purebred four-week-old male BALB/C nude mice were provided by the Shanghai SLAC Laboratory Animal Co., Ltd (Chinese Academy of Sciences, Shanghai, China). Huh-7 and HepG2 cells were injected subcutaneously into the right axilla (5 × 10^6 cells/100 μL/mouse). 40 mice for Huh-7 model and 20 mice for HepG2 model. Ten days after inoculation, the tumor formation rate was 100%, and the diameter of the xenograft tumors was approximately 0.8–1.0 cm. The mice in every cell model were randomly divided into four groups (AdSVPE1a-lncR, AdSVPeGFP-lncR, Ad5-eGFP, and blank control group), n = 10 in each group for Huh-7 model and n = 5 in each group for HepG2 model. The virus treatment groups received the corresponding viruses via intratumoral injection at a dose of 2 × 10^8 pfu/100 μL/mouse, once every other day for a total of five times. The blank control group received saline injections at the same time (100 μL/mouse). After treatments, the tumor size was measured every week and the tumor volume was calculated as follows: maximum diameter × minimum diameter^2 × 0.5. A growth curve was then plotted. When tumor volume in any group exceeded the allowed upper limit set by the animal experiment ethics committee, the experiment was stopped. The mice were sacrificed and the tumor specimens were weighed. Tumors were fixed with 10% neutral buffered formalin and the paraffin-embedded sections were used for IHC to determine the expression of miRNA target proteins (e.g., PTEN, p27kip1, CDK4, p38/MAPK and Survivin). The sections were also used for the labeling of apoptosis by TUNEL (TdT-mediated dUTP nick end labeling). Positive cells were counted in five fields in each slide under a 20× objective, and the statistical analysis was then performed.

HCC patient–derived xenograft tumor models in nude mice

HCC specimens were collected from 5 patients undergoing clinical operation during March 2015. A part of fresh HCC tissues was placed in DMEM cell culture medium containing 10% FBS for transplantation to the animal model, another part of tumor tissues was prepared for detection of miRNA expression by qRT-PCR and miRNA target gene expression by IHC. Fifteen healthy purebred BALR/C nude mice, male, 4 weeks old, were purchased from the Shanghai SLAC Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). The fresh tumor tissues were quickly cut to a depth of 2 mm in each piece and subcutaneously buried in mouse right axilla using a trocar puncture. The tumor tissue from each patient was transplanted to 3 nude mice, which were assigned to three experimental groups (AdSVPE1a-lncR, Ad5-eGFP, and blank control groups), that is, the 5 tumor xenografts of the 5 nude mice in each experimental group represented the 5 patients. Twenty days after inoculation,
the tumor xenografts were established, then the mice in every group were treated as aforementioned and the tumor growth was measured regularly. The tumors were removed and prepared for detection of miRNA expression by qRT-PCR and miRNA target gene expression by IHC.

Statistical analysis
The experimental data from three times of independent in vitro experiments, as well as the in vivo experimental data, were presented as "mean ± SD" and analyzed by one-way ANOVA. When the data were statistically different among the multiple groups, the SNK-q test was used to conduct the multiple comparison. The statistical analysis software package PASW Statistics 18 was used. The P value less than 0.05 was considered statistically significant.

Accession number
Human genome microarray data and human miRNA microarray data are deposited at the Gene Expression Omnibus database with an accession number GSE71108 (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE71108).

Results
Selective replication of adenovirus in HCC cells
The tumor-selective replicating adenovirus, AdSVPE1a-IncR, which expresses IncRNAi, uses the highly tumor-specific Survivin promoter to regulate the expression of E1a to achieve the targeted specifically replicating of virus (Fig. 1A). All of the HCC cell lines were Survivin-positive, and the strongest expression of Survivin occurred in HepG2, Hep3B, MHCC97H, Huh-7, and PLC/PRF/5 cells. There was no Survivin expression in the normal liver cells, WRL-68 and L02. After the cells were infected with AdSVPE1a-IncR, strong E1a expression was found in HepG2, Hep3B, MHCC97H, Huh-7, PLC/PRF/5, SMMC-7721, and MHCC97L cells, but BEL-7402, WRL-68, and L02 cells had a weaker E1a-positive expression (Fig. 1B). Consistent with the Survivin expression levels, the specific replication ability of AdSVPE1a-IncR was strong in HepG2, Hep3B, MHCC97H, Huh-7, and PLC/PRF/5 cells, with the highest value reaching 68,465.66-fold (Huh-7). The specific replication ability reached several hundred folds in SMMC-7721 and MHCC97L cells, whereas the replication in the normal cells, L02 and WRL-68, was not significant. AdSVPeGFP-IncR did not have significant replication activities in all HCC cells.

Figure 2.
Adenovirus-expressed IncRNAi protects the OncomiR target genes in HCC cells. A, cells were infected with virus AdSVPeGFP-IncR at an MOI of 1 pfu/cell and harvested for detecting IncRNAi expression by RT-PCR. B, qRT-PCR was used to detect IncRNAi expression. ***, P < 0.001. C, the expression of PTEN was detected by Western blot analysis in HepG2 cells after inhibition of miR21 and/or miR17-5p by their specific inhibitors and after infection of AdSVPE1a-IncR. GAPDH was used to normalize the relative expression levels. D, the sequence (5'-TCTGATAAGCTAGTAAGCACTTTA-3') containing the binding sites of miR21 and miR17-5p was used to construct the luciferase vector pGL3-miR21/17-5p. The luciferase assay was performed in HepG2 and L02 cells after being transfected with the inhibitors of miR21 and miR17-5p and infected with AdSVPE1a-IncR. The relative luciferase activity of pGL3-miR21/17-5p was normalized with the data of positive control pGL3- control. ***, P < 0.001; **, P < 0.01; and *, P < 0.05 versus the parental cell group.
and normal liver cells, with a difference less than 30-fold at 72 hours (Fig. 1C). Images acquired with the fluorescence microscope showed that there were scattered eGFP-positive cells in the AdSVPeGFP-lncR-infected HCC cells. The normal liver cells were all eGFP-negative. When the MOI was 100 pfu/cell, the AdSVPeGFP-lncR infection rate in experimental cells was as high as 80% (Fig. 1D).

**Targeted gene expression of adenovirus in HCC cells**

The encoding gene for IncRNAi contains a dozen of OncomiR complementary sequences and the sequences were repeated six times, therefore, by qRT-PCR amplification, AdSVPE1a-lncR-mediated IncRNAi expression resulted in 6 fragments with different molecular size (Fig. 2A). AdSVPE1a-lncR-mediated IncRNAi expression levels were high in Huh-7, Hep3B, and

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**Figure 3.**

Adenovirus-mediated IncRNAi expression inhibits HCC cell proliferation and mobility. A, cells were infected with viruses at MOIs of 1 to 500 pfu/cell. After 48 hours, MTT method was used to detect cell viability. B, cells were infected with viruses at an MOI of 1 pfu/cell and seeded in Transwell. After 48 hours, cells were scraped off the top chamber and stained with 0.1% crystal violet for 20 minutes. The number of viable cells was randomly imaged under a light microscope within three high-power fields (20 × objective lens). When cell invasion ability was monitored, a polycarbonate membrane (Matrigel) was added to the top chamber of the Transwell. Bar, 50 μm; *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus the blank control.
Figure 4.
Adenovirus-mediated IncRNAi expression affects HCC cell-cycle progression and apoptosis. A. cells were infected with viruses at an MOI of 1 pfu/cell and harvested after 48 hours, fixed with prechilled 75% ethanol, and stored at 4°C overnight. Cells were then washed twice with PBS, and RNase-containing propidium iodide (Pi) solution was added and incubated for 30 minutes in darkness. The cell cycle was analyzed via FCM. *, P < 0.05; **, P < 0.01 and ***, P < 0.001 versus the blank control. (Continued on the following page.)
HepG2 cells, followed by PLC/PRF/5, MHCC97H, BEL-7402, MHCC97L, and SMMC-7721 cells. The lncRNAi expression in the normal liver cells, WRL-68 and L02, was low, and the AdSVPeGFP-lncRNAi mediated lncRNAi expression was at a lower level in all experimental cells (Fig. 2B). To test whether lncRNAi exhibits its function by targeting OncomiRs, the expression of miR21 and miR17-5p in HepG2 cells was inhibited. Consequently, the expression of PTEN was upregulated by miR21 inhibitor or miR17-5p inhibitor, especially with the highest expression level in the cells transfected with double inhibitors, suggesting that PTEN is a target of both miR21 and miR17-5p. After infection of AdSVPE1a-lncRNAi, the PTEN expression was further increased, suggesting that lncRNAi could also suppress other molecules which were involved in the inhibition of PTEN expression and exert more effective anticancer efficacy (Fig. 2C).

The competitive inhibitory effect of lncRNAi was confirmed by the luciferase assay. The luciferase activity of pGL3-miR21/17-5p in HepG2 cells was lower than that in L02 cells. The miR21 inhibitor and miR17-5p inhibitor could increase the luciferase activity of pGL3-miR21/17-5p in HepG2 cells, but not the miR221 inhibitor. All inhibitors for miR21, miR17-5p, and miR221 could not affect the luciferase activity of pGL3-miR21/17-5p in L02 cells. After infecting with AdSVPE1a-lncRNAi, the luciferase activity of pGL3-miR21/17-5p was markedly

**Figure 4.**

(Continued.) B, adenovirus-mediated lncRNAi expression induces HCC cell apoptosis. Cells were infected with viruses at an MOI of 1 pfu/cell and harvested after 48 hours, and then stained with Annexin V/PI. The apoptotic rate was detected via FCM. *P < 0.05; **P < 0.01; and ***P < 0.001 versus the blank control.
increased in HepG2 cells, but not in L02 cells (Fig. 2D), demonstrating that the sequence (5'-TCTGATAAAGCTAGTAAGCACTTTA-3') within the luciferase vector pGL3-miR21/17-5p can be specifically bound by both miR21 and miR17-5p in HCC cells, and the lncRNAi expression can protect the target gene from being inhibited by OncomiRs.

Figure 5.
Adenovirus-mediated lncRNAi expression changes gene and miRNA expression profiles of HCC cells. Huh-7, HepG2, SMMC-7721, Hep3B, and L02 cells were seeded in a 24-well plate (1 × 10⁶ cells/well in 100 μL), infected with viruses at an MOI of 1 pfu/cell and harvested after 48 hours, and mRNA or miRNA was extracted. A, changes in mRNA expression were detected with the Agilent Whole Human Genome Microarray. The Venn diagrams showed the significant differentially expressed genes in the tested cells after infected with AdSVPE1a-IncR and AdSVPeGFP-IncR. B and C, the hierarchical clustering analysis (B) and volcano plot (C) of differentially expressed genes between the AdSVPE1a-IncR–infected HCC cells and their parental cells, AdSVPE1a-IncR– and AdSVPeGFP-IncR–infected HCC cells, AdSVPeGFP-IncR–infected HCC cells, and their parental cells. See also Supplementary Table S2. D, the expression of OncomiR representative target genes in HepG2 cells was detected by Western blot analysis. GAPDH was used as a loading control.

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Figure 5.
(Continued) E and F, changes in miRNA expression were detected with the Agilent Human miRNA Microarray. Hierarchical clustering analysis (D) and volcano plot (E) showed the differentially expressed miRNAs between the AdSVPE1a-lncR-infected HCC cells and their parental cells, AdSVPE1a-lncR-infected and AdSVPeGFP-lncR-infected HCC cells, and AdSVPeGFP-lncR-infected HCC cells and their parental cells. See also Supplementary Table S3. G, the expression levels of a dozen OncomiRs in HCC cells were detected by qRT-PCR. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Inhibitory effect of adenovirus-expressed lncRNAi on HCC cell proliferation and mobility
Cell proliferation experiments showed that AdSVPE1a-lncR had the strongest killing activity in Hep3B and Huh-7 cells. The viability of Hep3B cells was decreased to less than 50% when the adenovirus was added at an MOI of 0.5 pfu/cell and further decreased to less than 10% when the MOI was equal to 2 pfu/cell. The viability of Huh-7 cells was decreased to less than 50% when the MOI was equal to 1 pfu/cell and further decreased to less than 10% when the MOI was equal to 100 pfu/cell. AdSVPE1a-lncR also had a stronger killing activity in HepG2, MHCC97L, PLC/PRF/5, MHCC97H, and SMMC-7721 cells, as their viabilities were all less than 50% when
the virus was added at an MOI of 100 pfu/cell. AdSVPE1a-lncR had a weaker killing activity in BEL-7402 cells, as it decreased the viability to less than 50% at an MOI of 200 pfu/cell. AdSVPE1a-lncR did not have a significant impact on the proliferation of normal liver cells because the cell viability was greater than 80% when the MOI was equal to 500 pfu/cell. The positive control adenovirus, AdSVPeGFP-lncR, caused the viabilities of Hep3B, PLC/PRF/5, and Huh-7 cells to decrease to less than 50% when the adenovirus was added at an MOI of 200 pfu/cell, and the negative control adenovirus, Ad5-eGFP, did not have any significant inhibitory effect on any of the cells (Fig. 3A).

The Transwell cell migration and invasion experiments showed that AdSVPE1a-lncR had a stronger inhibitory effect on the cell migration and invasion ability of HepG2, MHCC97H, PLC/PRF/5, Hep3B, and Huh-7 cells compared with the blank control group, and this inhibitory effect on BEL-7402, SMCC-7721, and MHCC97L cells was relatively weaker. AdSVPeGFP-lncR also inhibited HCC cell migration and invasion of HepG2, MHCC97H, PLC/PRF/5, Hep3B, and Huh-7 cells, but its activity was significantly weaker than that of AdSVPE1a-lncR. AdSVPE1a-lncR and AdSVPeGFP-lncR did not significantly inhibit the mobility of normal liver cells (Fig. 3B).

**Adenovirus-expressed lncRNAi induces HCC cell-cycle changes and apoptosis**

Compared with the blank control group, there were no significant changes in each phase of the cell cycle when the normal liver cells were infected with the experimental adenovirus (AdSVPE1a-lncR) and control adenoviruses (AdSVPeGFP-lncR and Ad5-eGFP). When the HCC cells were infected with AdSVPE1a-lncR, the changes of cell-cycle phase varied in the different cell lines. PLC/PRF/5, Hep3B, and Huh-7 cells showed G0–G1 phase arrest with an increased proportion of cells in G0–G1 phase and a decreased proportion of cells in S-phase. PLC/PRF/5 and Hep3B cells also showed a reduced proportion of cells in G2–M phase. HepG2 and MHCC97H cells showed a reduced proportion of cells in G0–G1 phase, while the proportion of cells in S-phase was increased. Because the cells were unable to stride S-phase and were arrested in S-phase, therefore, the proportion of cells in G2–M phase was decreased. AdSVPE1a-lncR had a weaker effect on cell cycle in BEL-7402, SMCC-7721, and MHCC97L cells. SMMC-7721 cells showed an increased S-phase and a decreased G2–M phase. MHCC97L cells showed a decreased G2–G1 phase and an increased G1–M phase, and BEL-7402 cells only showed an increased G2–M phase (Fig. 4A).

After AdSVPE1a-lncR infection, all experimental HCC cells showed an increased proportion of apoptotic cells, with the most significant increase in Hep3B, HepG2, PLC/PRF/5, and Huh-7 cells, followed by MHCC97H, SMMC-7721, and MHCC97L cells. The increase in the proportion of apoptotic cells in BEL-7402, WRL-68, and L02 cells had no statistical significance. The ability of the nonreplicative adenovirus, AdSVPeGFP-lncR, to induce apoptosis was weaker, as its ability to increase the proportion of apoptosis had statistical significance only in Hep3B, PLC/PRF/5, and Huh-7 cells (Fig. 4B).

**Adenovirus-expressed lncRNAi affects gene expression profiles in HCC cells**

To explore whether AdSVPE1a-lncR has an effect on the expression profile of genes in HCC cell lines, we analyzed the expression profile of genes in HCC cell lines infected with AdSVPE1a-lncR and AdSVPeGFP-lncR using a genome-wide microarray for gene expression. The experimental results showed that there is a significantly differential expression of genes in HCC cell lines (Hep3B, HepG2, Huh-7, SMCC-7721) after infecting with AdSVPE1a-lncR, the differential expression level of genes in the four HCC cell lines is more obvious than that in normal liver cell line (L02; Fig. 5). Compared with the blank control cells, AdSVPE1a-lncR induced a differential expression of 2,091 genes, and AdSVPeGFP-lncR only affected 101 gene expressions in the four HCC cell lines (Fig. 5A). Among the differentially expressed 2,091 genes between the AdSVPeGFP-lncR–infected and blank control groups, 271 genes were upregulated and 176 genes were downregulated collectively in the four HCC cell lines. Particularly, the apoptosis induction genes and cell proliferation suppressor genes accounted for the large part of the upregulated genes (Supplementary Table S2). Hierarchical clustering analysis (Fig. 5B) and volcano plot (Fig. 5C) of differentially expressed genes between the AdSVPE1a-lncR–infected HCC cells and their parental cells, AdSVPE1a-lncR- and AdSVPeGFP-lncR-infected HCC cells, AdSVPeGFP-lncR–infected HCC cells and their parental cells showed that AdSVPE1a-lncR mediates bigger changes of gene expression level than AdSVPeGFP-lncR, involving into the upregulation of tumor suppressor genes and apoptosis induction genes. By Western blot analysis, the expression of OncomiRS representative target genes in HepG2 cells was detected and the results showed that the expression of tumor suppressors PTEN and p27Kip1 were increased, and the expression of oncogenic factors CDK4, p38/MAPK, and Survivin was decreased in HCC cells after lncRNAi expression, more obviously in the AdSVPE1a-lncR–infected HCC cells (Fig. 3D).

**Effect of adenovirus-expressed lncRNAi on miRNA expression profiles in HCC cells**

The miRNA expression microarray was used to detect the effect of the AdSVPE1a-lncR adenovirus on miRNA expression profile in...
HCC cells. The results showed that AdSVPE1a-lncR led to significant changes of miRNA expression profile in the four HCC cell lines, more obvious in Hep3B, HepG2, HuH-7 cells, but the changes were smaller in LO2 cells. There were 123 differentially expressed miRNAs between the AdSVPeGFP-lncR–infected HCC cells and blank control groups (Supplementary Table S3). Hierarchical clustering analysis (Fig. 5E) and volcano plot (Fig. 5F) showed that AdSVPE1a-lncR induced more differentially expressed miRNAs in the tested HCC cells compared with AdSVPeGFP–lncR. The qRT-PCR results showed that only miR21 was downregulated in all HCC cell lines after lncRNAi expression, the changes of other miRNAs were not coincident in the tested cell lines, for example, miR221 was decreased in Hep3B and SMMC-7721 cells and increased in HepG2 cells, and did not change in HuH7 cells (Fig. 5G).

**Antitumor effect of adenovirus-expressed lncRNAi on HCC cell line xenografts in nude mice**

On HuH7 and HepG2 xenograft tumors in nude mice, the tumor growth rate of the AdSVPE1a–lncR treatment group was significantly slower than that of the blank control group at 14 days after treatments. At 21 days, the tumor volume started to decrease. The AdSVPeGFP–lncR group also had some growth inhibition 28 days after treatment, and the difference was significant compared with the blank control group even though the tumor continued to grow. The Ad5-eGFP group did not show growth inhibition during the experiment. The observation continued till 42 days after treatment, and the tumor volume in the AdSVPE1a–lncR group was significantly smaller than that before treatment (Fig. 6A). After finishing the observation, the xenograft tumors of HuH-7 cell models were weighed and compared. The tumor weights in the AdSVPE1a–lncR group were significantly less than those of the other groups (Fig. 6B). The tumors were examined by TUNEL method and the results showed that the apoptotic rate of cancer cells in the AdSVPE1a–lncR group was significantly increased and there was also an increase to a lesser extent in the AdSVPeGFP–lncR group (Fig. 6C). The immunohistochemical staining for the expression of miRNA target genes showed that PTEN and p27kip1 expression was significantly increased in the AdSVPE1a–lncR group, while CDK4, p38/MAPK, and Survivin expression was significantly decreased. In AdSVPeGFP–lncR group, PTEN expression was increased and Survivin expression was decreased (Fig. 6D).

**Antitumor effect of adenovirus-expressed lncRNAi on HCC PDX models in nude mice**

The antitumor effect of oncolytic adenovirus-mediated lncRNAi expression on tumor xenografts was observed. After 28 days of viral therapy, compared with the blank control group, the AdSVPE1a–lncR group showed a significant decrease in tumor volume, but did not in the Ad5–eGFP group (Fig. 7A). The xenograft tumors were collected at day 28 after viral therapy. The xenografts, as well as the HCC clinical specimens, were used to detect the expression of OncomiRs and their target genes. The expression levels of miR21, miR221/222, miR224, miR17-5p, miR10b, miR106b, miR151-5p, miR155, miR181a/181b, and miR184 were significantly higher in the HCC clinical specimens and their PDX models (Fig. 7B). The expression of lncRNAi was markedly higher in the AdSVPE1a–lncR group (Fig. 7C). The expression of representative miRNA target genes showed that PTEN expression was significantly increased and Survivin expression was significantly decreased in the xenografts of the AdSVPE1a–lncR group compared with that of the Ad5–eGFP group and the primary HCC clinical specimens (Fig. 7D).

**Discussion**

HCC is a common malignant disease. An increasing number of studies have shown that many miRNAs are involved in the occurrence and progression of HCC by regulating the expression and function of their target genes. During the occurrence of HCC, miRNAs can act as OncomiRs or tumor-suppressive miRNAs. The upregulation of OncomiRs or the downregulation of tumor-suppressive miRNAs can promote the proliferation, invasion, and metastasis of cancer cells, conferring more malignant biologic properties to cancer cells. Studies have found that overexpression of miR21 in HCC can inhibit the expression of tumor suppressor gene PTEN (26, 27). PTEN is an important inhibitory protein of the PI3K/serine threonine protein kinase (AKT) pathway. Therefore, the consequence of the inhibition of PTEN by miR21 is the activation of the PI3K/AKT pathway, which promotes cancer cell proliferation and metastasis (28). miR221/222 acts on many key tumor suppressors, including Bmf (29), p27Kip1 (30), p57Kip2 (31), PTEN (32), tissue inhibitors of metalloproteinases (TIMP; ref. 33), and DNA damage-inducible transcript 4 (DDIT4; ref. 34), to mediate the occurrence and progression of tumors. miRNAs are abnormally expressed during the early oncogenesis of hepatitis B virus (HBV)–related HCC (35). Among the HBV-related miRNAs, miR21 and miR501-5p can promote HBV replication, thereby promoting the occurrence and progression of HCC (36–38). In contrast, the decrease of miR122 expression may reduce the antiviral ability of the body and mediate the pathogenesis of primary HCC (39). These miRNAs execute different biologic functions through acting on different target genes and different signal transduction pathways during the occurrence and development of HCC. These miRNAs can be categorized according to their functions as follows: miR21, miR221/222, miR224, miR17-5p/20a, miR10b, miR106b, miR151-5p, miR155, miR181a/181b, and miR184 can promote the proliferation, invasion, and metastasis of HCC cells (12–17); miR221/222, miR224, miR10b, and miR155 can inhibit HCC cell apoptosis (13, 18, 19, 21); and miR21, miR221/222, miR143, miR1, and miR501-5p can promote the replication of HBV, induce carcinogenesis, and enhance cancer cell proliferation (36–38).

In HCC tissue, the expression of miRNAs is abnormal, thus affecting the biologic behaviors of cancer cells, which provides a good target for HCC treatment. By interfering with miRNA expression to regulate the expression of oncogenes or tumor suppressor genes, we can achieve targeted treatment of HCC. For the OncomiRs that are highly expressed in HCC cells, we can use the antisense nucleotide technique or specific inhibitors to suppress miRNA activity to inhibit tumor growth (40). One of our studies has shown that miR21 can not only inhibit the expression of the tumor suppressor gene PTEN in HCC cells but also inhibit the expression and function of sulfatase 1 (hSulf-1). The decreased expression and the loss of function of PTEN and hSulf-1 can upregulate AKT/ERK activity, thereby enhancing the proliferation, invasion, and migration ability of HCC cells. Inhibition of miR21 function by miR21 inhibitor can inhibit the proliferation and metastasis capacity of HCC cells (28). However, compared with
normal cells, there are many differentially expressed miRNAs in cancer cells, and their functions are complex. During the occurrence and development of HCC, many miRNA molecules are involved, and these miRNAs regulate a broad spectrum of target genes or affect many signal pathways. Therefore, the interference of the expression of a single miRNA has a limited inhibitory effect on tumors, while joint interference of multiple miRNAs would be more effective. Thus, it is important to further discuss how to simultaneously silence multiple overexpressed OncomiRs to enhance the efficacy of intervention treatment of HCC.

Taking advantage of the principle that miRNAs regulate the expression of target genes, we designed a more optimal therapeutic strategy for HCC. This strategy uses the tumor-selective replicating oncolytic adenovirus, which is controlled by the Survivin promoter, as a vector to express an artificially synthesized IncRNAi. This IncRNAi contains a tandem of sequences that are complementary to seed sequences of 12 OncomiRs, including miR21, miR221/222, miR224, miR17-5p/20a, miR10b, miR106b, miR151-5p, miR155, miR181a/181b, miR184, miR1, and miR501-5p, all of them overexpress in HCC and can promote the occurrence and development of HCC cells through multiple mechanisms. The high expression of IncRNAi mediated by tumor-selective replicating adenovirus competes with the mRNAs of OncomiR target genes to bind to the OncomiRs inside cancer cells, thereby consuming the intracellular OncomiRs to protect OncomiR target genes, to fully protect multiple tumor suppressor genes and to exert greater anticancer efficacy. For this treatment strategy, only

Figure 7.
Adenovirus-mediated IncRNAi expression inhibits tumor growth of HCC PDX xenografts in nude mice. A, fresh HCC tissues from 5 patients were cut to a depth of 2 mm in diameter and subcutaneously buried in the right axilla of 15 nude mice. After tumor xenografts were formed, mice in the Ad5SVPE1a-IncR and Ad5-eGFP groups were intratumorally injected the corresponding viruses at a total of 1 × 10^9 pfu per mouse. The blank control group was injected with the viral preservation solution instead of virus injection. Tumor volume was measured regularly; ***, P < 0.001 versus the blank control group. B, the xenografts, as well as the HCC clinical specimens, were used to detect the expression of OncomiRs by qRT-PCR; C, the xenografts were used to detect the expression of IncRNAi by qRT-PCR; ***, P < 0.001 versus the blank control group. D, the xenografts, as well as the HCC clinical specimens, were used to detect the expression of OncomiR target genes, PTEN and Survivin, by IHC. The positive cell percentages within five high-power fields (20 × objective lens) on each section were calculated under microscope. Bar, 100 μm; ***, P < 0.001 versus the blank control group and the primary tumors.
when the copy number of the competitor (i.e., IncRNAi) is significantly higher than that of the target being competed (i.e., target gene mRNAs), the competitor can more effectively occupy the dominant position of the competition and greatly consume OncomiRs. We used the tumor-selective replicating adenovirus as the vector to mediate the expression of IncRNAi. Because the tumor-selective replicating adenovirus can specifically replicate in large amounts in cancer cells and lyse cancer cells, the destroyed cancer cells release progeny viruses to continuously infect more cancer cells. Taking advantages of the specific replicating ability in high copy numbers, the high dispersion ability and high transfection ability of the tumor-selective replicating adenovirus in cancer cells, the copy number of transgene carried by this type of virus will be increased exponentially with the viral replication (41). The IncRNAi, which is highly expressed in high copy number in cancer cells, can increase its competition effectiveness for binding to OncomiRs so that it can fully protect multiple target genes of OncomiRs and inhibit the progression of HCC.

Studies have found that adenoviruses regulated by the highly tumor-specific Survivin promoter can replicate by many thousand-fold in the Survivin-positive HCC cells, while mediating IncRNAi expression in high copy number and high expression efficiency. In normal cells, however, the virus does not replicate and the expression levels of IncRNAi are low. Highly expressed IncRNAi in HCC cells can effectively inhibit cell proliferation, invasion, and migration ability, and can induce apoptosis and cell-cycle changes. The action of IncRNAi was the most significant in Hep3B, Huh-7, HepG2, MHCC97H, and PLC/PRF/5 cells, which have high expression levels of Survivin. In contrast, the expression of IncRNAi mediated by the nonreplication viral vector had low copy numbers, resulting in a weaker effect on cancer cells. Through microarray detection, we found that the protein and miRNA expression profiles in the IncRNAi-expressed cancer cells significantly changed. The upregulated genes were mainly apoptosis induction genes and tumor suppressor genes. These changes were also accompanied by expression changes in many genes involved in the regulation of other biologic properties. These results suggested that the high copy number expression of IncRNAi has a protective effect on the target genes of OncomiRs. We further verified the therapeutic efficacy of this anticancer strategy in HCC xenograft tumor models in nude mice, in which the IncRNAi competitively consumes OncomiRs. The experiments confirmed that this therapeutic method can completely inhibit tumor growth and even shrink tumors.

In summary, the anticancer strategy, which we proposed to competitively consume OncomiRs by IncRNAi, can simultaneously and effectively block the functions of multiple OncomiRs that have different or complementary mechanisms. This approach can overcome the defects of single miRNA interference. Moreover, the Survivin promoter regulates adenoviruses to specifically replicate in cancer cells, and the action of the expressed IncRNAi is only limited to OncomiRs and is not active on other tumor-suppressive miRNAs. Normal cells are not affected because OncomiRs are not expressed or their expression level is extremely low in normal cells. Therefore, the anticancer therapeutic efficacy of this strategy is increased and the safety is improved. In this artificially designed and synthesized IncRNAi, the translation stop codon TAG is introduced at its beginning and ending position to block its translation, which further ensures its safety. Thus, this strategy has established a technology platform with a reliable therapeutic effect for the treatment of tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Li, Y. Su, C. Su
Development of methodology: X. Li, B. Sun, W. Ji, Y. Xu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Sun, W. Ji, Y. Xu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Sun, Z. Peng
Writing, review, and/or revision of the manuscript: X. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Su, C. Su
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