Interfering RNA with multi-targets for efficient gene suppression in HCC cells

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Abstract. RNA interference (RNAi) technology has been widely used in therapeutics development, especially multiple targeted RNAi strategy, which is a better method for multiple gene suppression. In the study, interfering RNAs (iRNAs) were designed for carrying two or three different siRNA sequences in different secondary structure formats (loop or cloverleaf). By using these types of iRNAs, co-inhibition of survivin and B-cell lymphoma-2 (Bcl-2) was investigated in hepatocellular carcinoma (HCC) cells, and we obtained promising gene silencing effects without showing undesirable interferon response. Furthermore, suppression effects on proliferation, invasion, and induced apoptosis in HCC cells were validated. The results suggest that long iRNAs with secondary structure may be a preferred strategy for multigenic disease therapy, especially for cancer and viral gene therapy and their iRNA drug development.

Introduction

RNA interference (RNAi) has shown tremendous potential in medicinal therapeutics development in past decade (1-4). Now, it has been confirmed that RNAi has great potential in treatment of various human diseases, such as viral infections (5,6), cancers (7-10) and orphan disease (11). Recently, an attempt using a long double-strand siRNA was made, as an alternative way, to carry on multiple interfering RNAs (iRNA) for multiple gene suppression for treatment of many human diseases (12-14), such as therapy for hepatocellular carcinoma (HCC) (10). Currently there are more than thirty types of siRNA drug candidates entered into the stage of clinical trials (15-17). However, these synthetic therapeutic long siRNA constructs in literature, including our previously study (18), were all based on a primary structure format (linearlized). It is a well-known fact that the longer the RNA molecule is, the easer the degradation occurs during RNA processing in circulation or in cell level.

To stabilize long therapeutic RNA, in the study we first described a group of long synthetic iRNA with multiple siRNA targets in secondary structures (loop or cloverleaf) and applied them to HCC cells for multiple gene suppression.

Materials and methods

Cell lines and cell culture. SMMC-7721 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). These cell lines were purchased from the Institute of Cell Biology, Chinese Academy of Sciences, and cells were maintained at 37˚C with 5% CO_2 and 95% air in a humidified incubator.

Multi-target iRNA format design and transfection. The gene sequence of survivin (accession no. NM_001168) and B-cell lymphoma-2 (Bcl-2) (accession no. NM_000633.2) were obtained from National Center of Biotechnology Information (NCBI) GenBank (USA), and the single target siRNA (19 bp duplex with 2-nt 3'-overhangs) targeting either survivin or Bcl-2 were screened in our precious studies (Table I) (18,19). A double-stranded RNA sequence with no homology with human genes was designed as siRNA negative control (NC_siR). All RNA oligonucleotides were synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, China). The multi-target iRNA constructs targeting survivin and Bcl-2 were designed according to the principle of Biomics Biotechnologies Co., Ltd. (Table II). The cells were transfected *in vitro* with siRNAs using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific) according to the instructions of the manufacturer.

Real-time quantitive PCR (RT-qPCR). Total RNA of cells were extracted by RISO™ RNA extraction reagent (Biomics Biotechnologies Co., Ltd.) and then performed in a RT-qPCR reaction: 12.5 μl of 2X One-Step qPCR Mix (Biomics Biotechnologies Co., Ltd.), 0.5 μl of each forward and reverse

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Table I. Sequences of the single target siRNAs.

siRNAs		Primer sequences (5'-3')	
Survivin siRNA1	S2	Sense	GACUUGGCCCAGUGUUUCUdTdT
		Antisense	AGAAACACUGGGCCAAGUCdTdT
Survivin siRNA2	S38	Sense	UCCUUUCUGUCAAGAAGCAGUUdTdT
		Antisense	AACUGCUUCUUGACAGAAAGGAdTdT
Bcl-2 siRNA	B2	Sense	GGAUGACUGAGUACCUGAAdTdT
		Antisense	UUCAGGUACUCAGUCAUCCdTdT
Negative control siRNA	NC_siR	Sense	UUCUCCGAACGUGUCACGUdTdT
		Antisense	ACGUGACACGUUCGGAGAAdTdT

NC_siR, siRNA negative control; Bcl-2, B-cell lymphoma-2.

Table II. Sequences of RT-qPCR primers.

Gene name	Primer sequences (5'-3')
Survivin	F: CGACGTTGCCCCCTGCCTG
	R: AAGGAAAGCGCAACCGGACGA
Bcl-2	F: GGCTGGGATGCCTTTGTG
	R: GCCAGGAGAAATCAAACAGAGG
Ago2	F: GGCAGGAAGAATCTATACAC
	R: CTTGATGGACACCTTGAAG
OAS1	F: GTGAGCTCCTGGATTCTGCT
	R: TGTTCCAATGTAACCATATTTCTGA
IFIT1	F: AATAGACTGTGAGGAAGGATGG
	R: TCCAGGCGATAGGCAGAG
GAPDH	F: GAAGGTGAAGGTCGGAGTC
	R: GAAGATGGTGATGGGATTTC

Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

primers (10 μM each; Biomics Biotechnologies Co., Ltd.), 0.5 μl of 50X SYBR-Green I and 4 μl total RNA was then subjected to reverse transcription for 30 min at 42˚C and initially denatured at 95˚C for 5 min, and then to 45 cycles of amplification with the condition of 95˚C denaturation for 20 sec, 55˚C annealing for 30 sec, and 72˚C extension for 30 sec. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. The experiment was performed in triplicate. All primer sequences are shown in Table II. The results were analyzed by $2^{-\Delta\Delta Ct}$ method (20).

Western blotting. Cells were plated in a 6-well plate at $1x10^6$ cells/well and grown for 24 h until ~70-80% confluence. After cells were treated as described above for 48 h, they were harvested and lysed in RIPA buffer (Pierce, Rockford, IL, USA). Total protein was quantified by bicinchoninic acid (BCA) assay (Promega, Madison, WI, USA), and then ~20 μg of protein was separated by polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinylidine difluoride filter (PVDF) membranes (Millipore, Billerica, MA, USA), followed by blocking with 5% skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) buffer for 2 h at room temperature, then incubated with rabbit anti-human survivin antibody (1:500 dilution) and mouse anti-human Bcl-2 (1:500 dilution), mouse anti-human β-actin antibody (1:500 dilution) (all from Abcam, Cambridge, MA, USA) as internal control. The membranes were washed in TBST and incubated with a goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibody (1:1,000 dilution; Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 2 h. Then, the specific proteins were detected with ECL chemiluminescence reagent (Beyotime, Beijing, China); the membranes were exposed to film (Kodak, Rochester, NY, USA).

Cell viability assay. MTT assay was used to measure the viability of cells. Cells were plated into a 96-well plate at $5x10^3$ cells/well and grown for 24 h, then treated as above for 0, 24, 48, 72 and 96 h, 10 μl MTT (Beyotime) were added to each well of 96-well plate containing 100 μl DMEM medium and incubated at 37˚C for 4 h, then 150 μl/well DMSO was added and incubation was continue at 37˚C for 10 min. The optical density (OD) was measured at 490 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Cell invasion assay. Cells were seeded into a 24-well plate at $2x10^4$ cells/well and grown for 24 h, then treated as described above, post 48 h treament, cells were suspended in DMEM medium at the density of $1x10^6$ cells/ml. Briefly, Transwell chambers (Corning, Inc., Corning, NY, USA) and treated with DMEM for 1 h before treatments; an 8 μm pore polycarbonate membrane was coated with 50 μl of 0.5 mg/ml Matrigel (BD Biosciences, San Jose, CA, USA) and used to separate the upper and lower chambers. Cell suspension (100 μl) was added into each upper chamber and 600 μl DMEM containing 10% FBS or conditioned medium which was the cell supernatant with siRNAs post-transfected for 48 h. The cells on the top surface of the membrane were carefully removed at 24 h post treatments. Cells on the Transwell chambers were fixed for 30 sec in 10% formaldehyde, and then stained by 0.5% crystal violet, after washing by phosphate-buffered saline (PBS), the cells on the top surface of the membrane were carefully removed again. The cells on the bottom surface of the membrane were counted in 3-5 random fields under a microscope (magnification, x100).

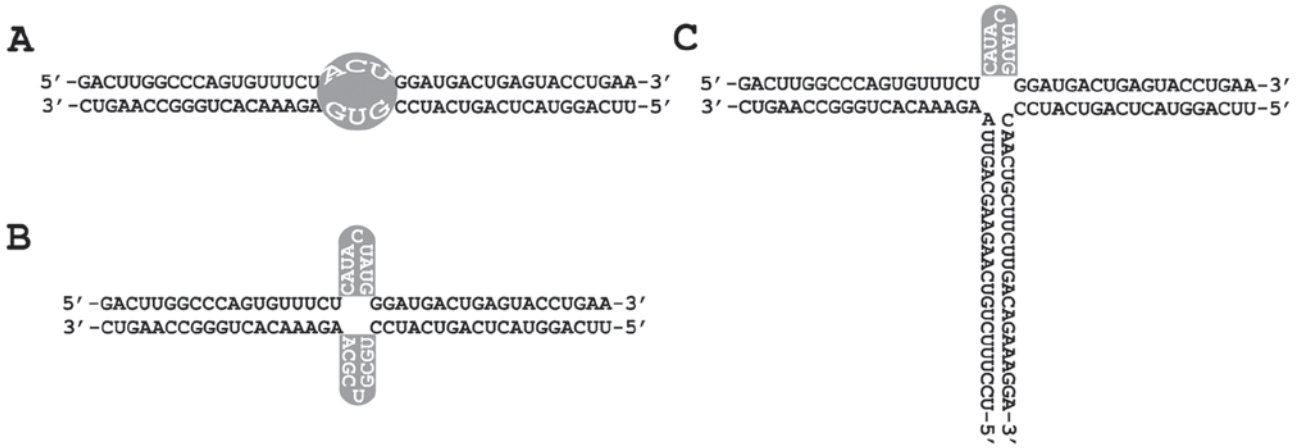

Figure 1. The structures of different multi-target interfering RNAs (mtg_iRNA). (A) mtg_iRNA1; (B) mtg_iRNA2; (C) mtg_iRNA3.

Cell apoptosis assay. Annexin V-FITC/PI double staining and flow cytometry (FCM) analysis method was used to determine cell apoptosis. Briefly, $1x10^6$ cells/well in a 6-well plate treated with different treatments as above for 48 h were harvested and washed in PBS, cells were stained by using Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA), then detected by FCM analysis (BD Biosciences).

Statistical analysis. All experiments were performed independently three times, the results are shown as mean ± standard deviation (SD), and statistical analyses were performed using SPSS 19.0 software. The differences were compared using Student's t-test and one way ANOVA followed by post hoc test to assess statistical significance. All P-values were based on a two-sided statistical analysis and P<0.05 was considered to indicate statistical significance.

Results

Designing different multi-target iRNAs. Multi-target iRNAs targeting survivin and Bcl-2 were designed according to different structure formats (Fig. 1). The sense and antisense strands of iRNAs were synthesized by Biomics Biotechnologies Co., Ltd. and iRNAs were obtained after annealing of both strands (Table II and Fig. 1).

Inhibition effects of target genes in HCC cells by multi-target iRNAs. The result of RT-qPCR and western blot analysis showed that, compared with untreated cell, the mRNA and protein level of survivin were both inhibited by survivin siRNA (S2 or S38). The mRNA and protein level of Bcl-2 were both inhibited by Bcl-2 siRNA (B2) (P<0.05) (Fig. 2).

Compared to untreated group, the mRNA level of survivin was inhibited by multi-target interfering RNAs (mtg_iRNA1), mtg_iRNA2 and mtg_iRNA3 up to 45, 27 and 53% (P<0.05) (Fig. 3A); the mRNA level of Bcl-2 was inhibited up to 38, 27 and 33% (P<0.05) (Fig. 3B); and there was no difference between NC_siRNA and untreated ones. Moreover, mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 showed no interferon response (Fig. 3C). The protein level of survivin was inhibited by mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 up to 48, 46 and 68%, respectively (P<0.05); the protein level of Bcl-2 was inhibited up to 45, 57 and 48% (P<0.05), and there was no difference between NC_siR and untreated group (P>0.05) (Fig. 3D).

Gene knockdown by mtg_iRNAs in an Ago2-dependent manner. Long double-stranded RNA was reported with gene knockdown effect in an Ago2-dependent manner (21). To verify the manner of the effect of mtg_iRNAs, the Ago2 siRNA (sense, 5'-AAUCUCUUCUUGCCGAUCGdTdT-3' and antisense, 5'-CGAUCGGCAAGAAGAGAUUdTdT-3') was used to knockdown the expression of Ago2, and we further investigated whether mtg_iRNAs could downregulate the target genes. As shown in Fig. 3, compared with untreated cells, the expression of Ago2 was inhibited efficiently by Ago2_siR, when Ago2 was downregulated, target gene survivin or Bcl-2 could not be inhibited by mtg_iRNAs, and thus gene knockdown by mtg_iRNAs was Ago2-dependent (Fig. 3E).

Inhibition effects on cell viability by multi-target siRNAs. The viability of SMMC-7721 cells was measured by MTT assay. The absorbance values (490 nm) of the cells at 48, 72 and 96 h post-transfection with mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 were significantly lower than those of NC_siR treated and untreated cells, respectively (P<0.05) (Fig. 4). There was no significant difference between NC_siR treated and untreated cells (P>0.05).

Inhibition effects on cell invasion by multi-target siRNAs. The cell invasion abilities with different treatments were detected by Transwell assay, the result showed that the invasion of the cells treated with mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 were inhibited significantly post 48 h transfection (P<0.05). There was no significant difference of inhibition abilities between NC_siR and untreated cells (P>0.05) (Fig. 5).

Cell apoptosis induces multi-target siRNAs. Annexin V-FITC/PI double staining and FCM analysis were used to detect the ability of mtg_iRNAs on inducing SMMC-7721 cell apoptosis. The result showed that cells treated with mtg_iRNA1, 20.52±0.81%, mtg_iRNA2, 22.36±1.15% and mtg_iRNA3,

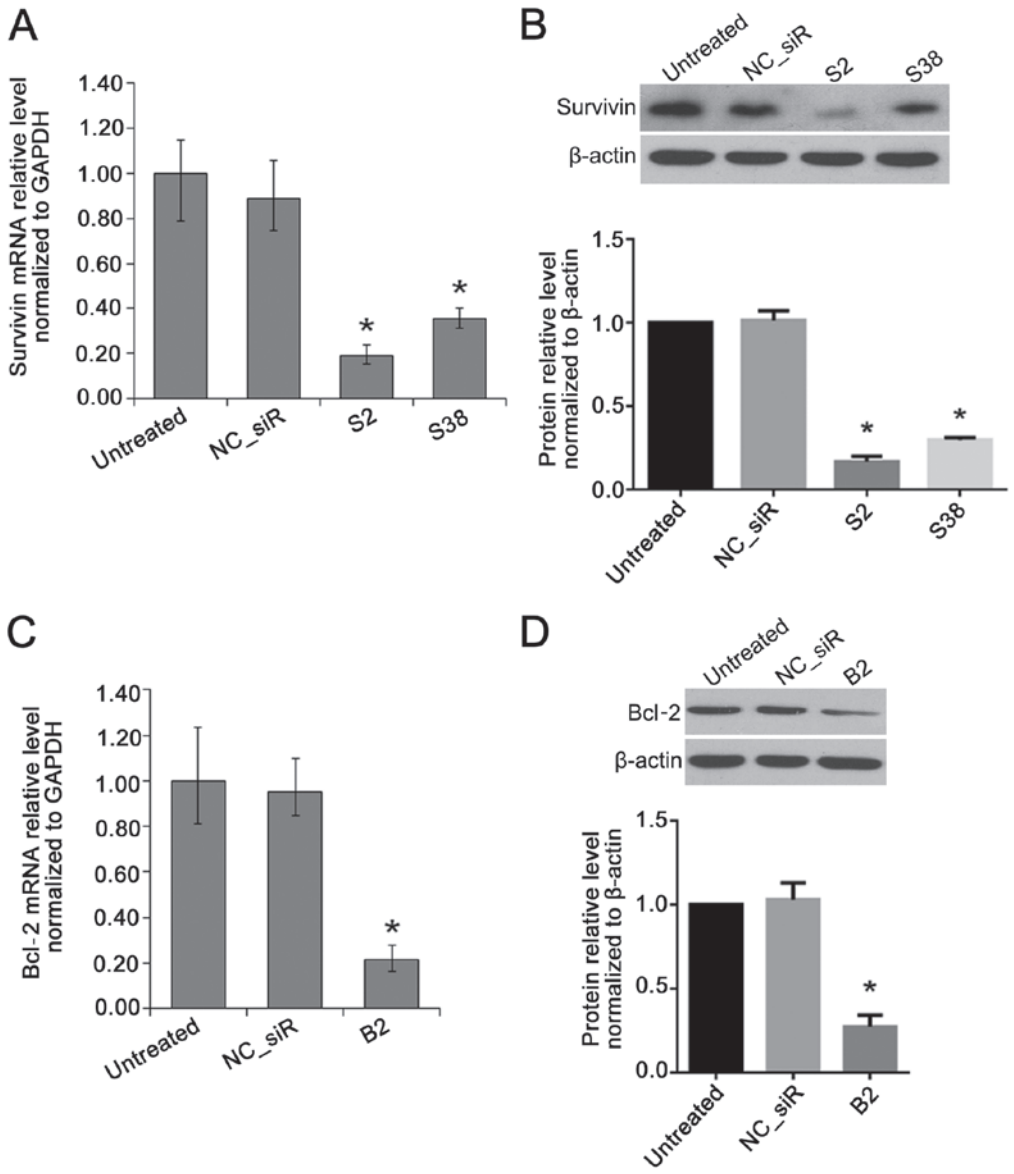

Figure 2. Inhibition effects on survivin or Bcl-2 by single target siRNA in hepatocellular carcinoma (HCC) cells. (A) The mRNA expression level of survivin inhibited by S2 or S38 siRNA detected by RT-qPCR, *P<0.05 vs. untreated cells. (B) The mRNA expression level of Bcl-2 inhibited by B2 siRNA detected by RT-qPCR, *P<0.05 vs. untreated cells. (C) The protein expression level of survivin inhibited by S2 or S38 siRNA detected by western blotting, *P<0.05 vs. untreated cells. (D) The protein expression level of Bcl-2 inhibited by B2 siRNA detected by western blotting, *P<0.05 vs. untreated cells.

38.79±1.63% resulted in a significant increase of apoptosis compared with that of NC_siR treated cells, 1.96±1.35% and untreated cells, 1.52±1.41% (P<0.05) (Fig. 6).

Discussion

The present study is the first attempt to use long synthetic iRNA formats (loop or cloverleaf) to carry multiple siRNA targets. Survivin and Bcl-2 were used as HCC therapeutic targets in our study for iRNA therapeutics development. survivin is an apoptosis inhibitor that is expressed during the G2/M phase of the cell cycle and it is a member of the inhibitor of apoptosis (IAP) gene family, survivin encodes negative regulatory proteins that prevent apoptotic cell death (25). Bcl-2 is specifically considered as an important anti-apoptotic oncogene, and it is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (26,27).

The efficiency of gene co-inhibition by long secondary structure iRNA was demonstrated (Fig. 3A, B and D), in which all three mtg_iRNAs inhibited target genes (survivin or Bcl-2) effectively in both mRNA and protein level.

The side-effect of undesired interferon response caused long double-stranded RNA (dsRNA) is a major concern. It was addressed by a previous study indicating that specific gene knockdown via RNAi triggered by long dsRNA could induce interferon response (28), thus the expression of OAS1 and IFIT1 were detected to monitor the interferon response and a synthetic dsRNA analog-poly(I:C) was used as positive control (29). The designed long secondary structure iRNAs here had no obvious interferon response in mammalian cells (Fig. 3C). RNAi effects can be triggered by different structural modifications of siRNA, also long multi-target based siRNA structures designed as Dicer substrates have been developed in therapeutic research (19), and these siRNAs

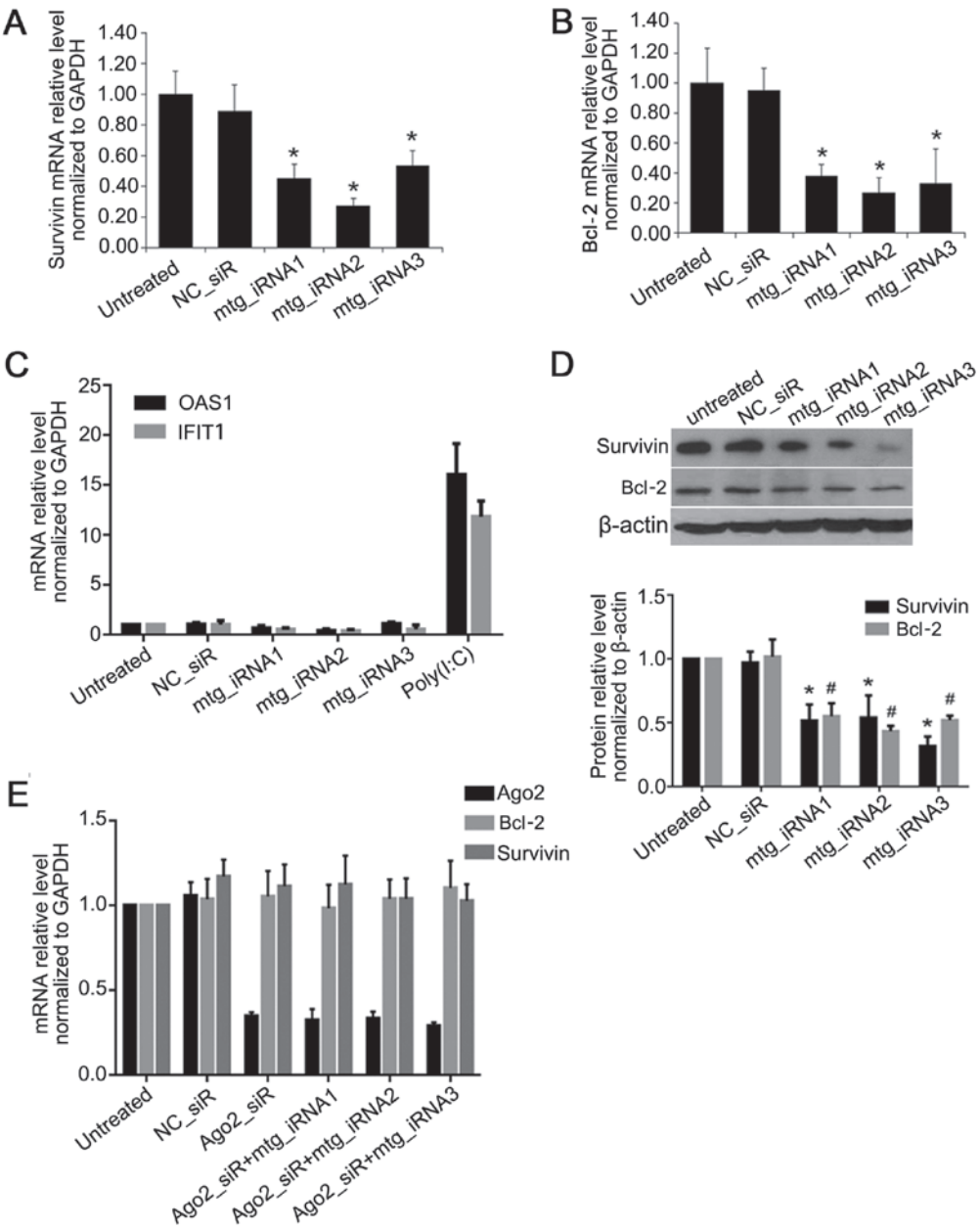

Figure 3. Inhibition effects on survivin or Bcl-2 by multi-target interfering RNAs (mtgRNAs) in hepatocellular carcinoma (HCC) cells. (A) The mRNA expression level of survivin inhibited by mtg_iRNA1, mtg_iRNA2 or mtg_iRNA3 detected by RT-qPCR, *P<0.05 vs. untreated cells. (B) The mRNA expression level of Bcl-2 inhibited by mtg_iRNA1, mtg_iRNA2 or mtg_iRNA3 detected by RT-qPCR, *P<0.05 vs. untreated cells. (C) No interferon response induced by mtg_iRNA1, mtg_iRNA2 or mtg_iRNA3. (D) The protein expression level of survivin or Bcl-2 inhibited by mtg_iRNA1, mtg_iRNA2 or mtg_iRNA3 detected by western blotting, *P<0.05 the protein level of survivin and #P<0.05 the protein level of Bcl-2 vs. untreated cells. (E) The mRNA expression level of survivin or Bcl-2 by mtg_iRNAs when Ago2 was downregulated.

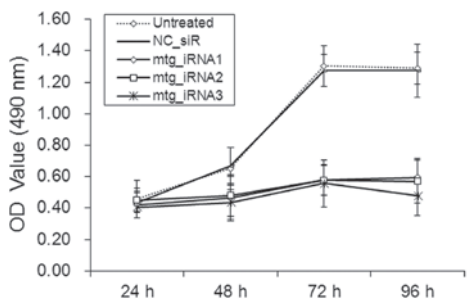

Figure 4. Inhibition effects on cell viability by multi-target interfering RNAs (mtg_iRNAs) at 0, 24, 48, 72 and 96 h detected by MTT assay. The viabilities of cells at 48, 72 and 96 h were inhibited significantly by mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 compared to those of NC_siR treated and untreated cells.

were all Ago2-dependent. In the present study, also our mtg_iRNAs were all Ago2-dependent by the method of Ago2 downregulation first (Fig. 3E). Furthermore, we demonstrated that the proliferation and invasion of HCC cells were inhibited, also cell apoptosis was promoted by mtg_iRNAs effectively (Fig. 4-6). The results of this study demonstrated that the designed mtg_iRNAs construct had RNAi activities on knockdown target genes simultaneously. The results showed that, mtg_iRNAs may be a preferred strategy for multigenic disease therapy, especially in cancer.

In conclusion, the long iRNA with different formats (loop and cloverleaf) showed initial multi-gene co-inhibition effects without showing side-effect of interferon

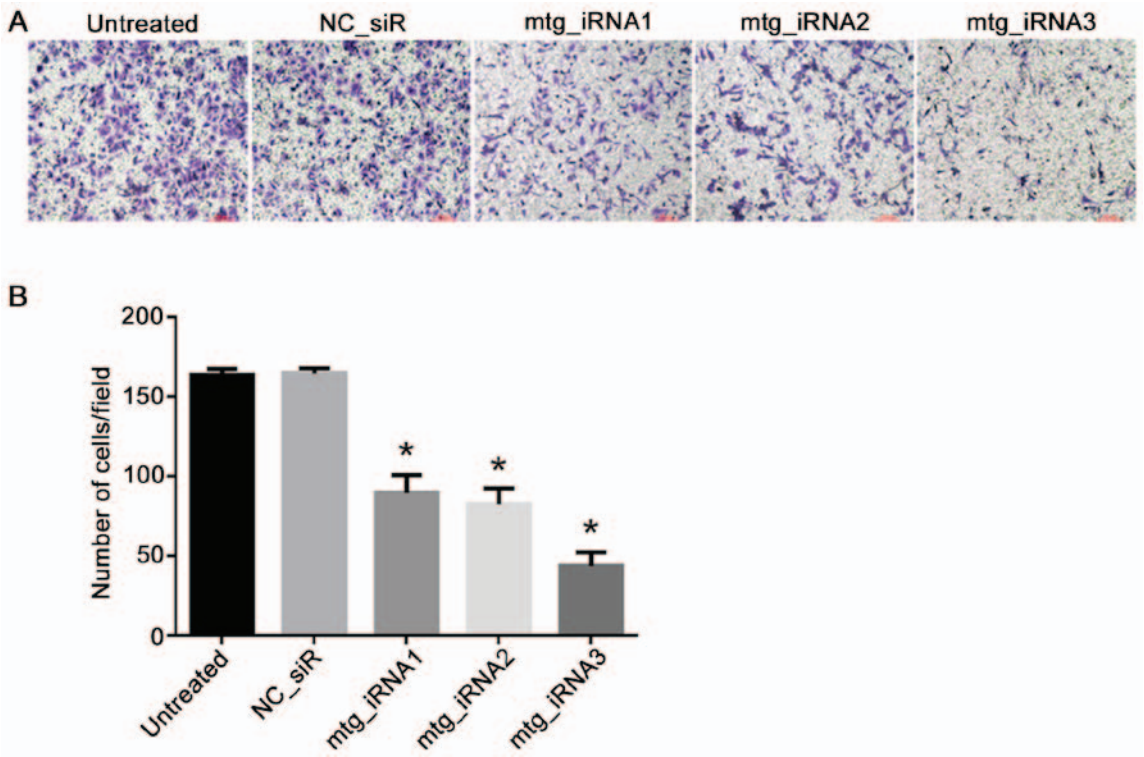

Figure 5. Inhibition effects on cell invasion by multi-target interfering RNAs (mtg_iRNAs) detected using Transwell chamber assay. (A) The representative figures of cells with different treatments detected by Transwell chamber assay. (B) The invasions of cells were inhibited significantly by mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 ($P<0.05$).

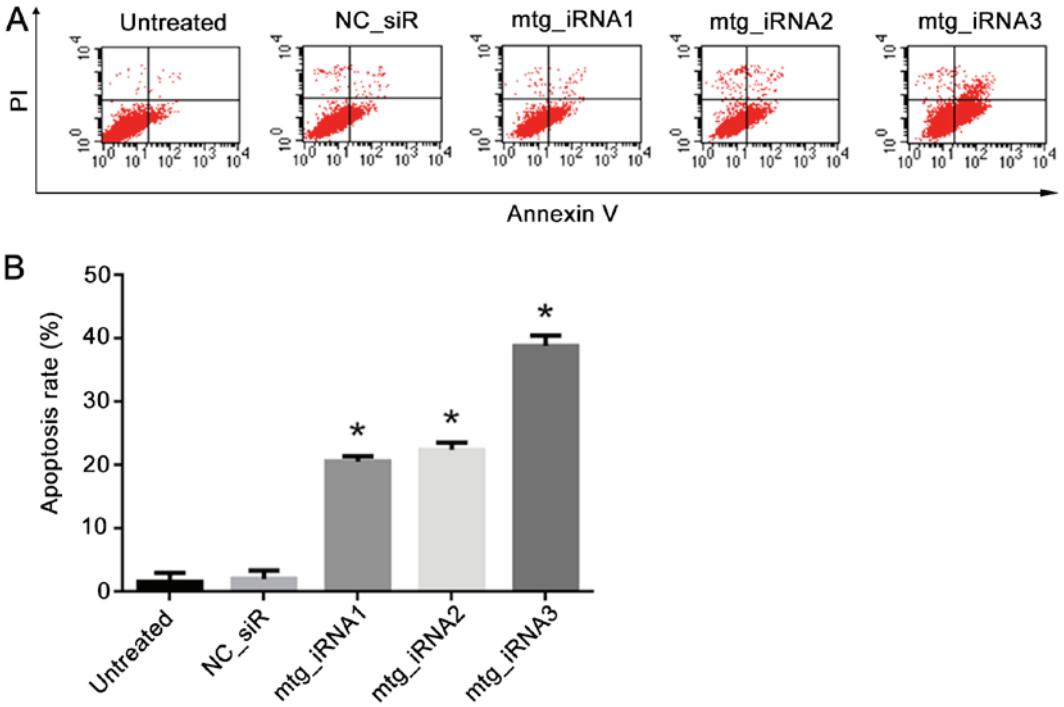

Figure 6. Effects on cell apoptosis by multi-target interfering RNAs (mtg_iRNAs) measured by flow cytometry (FCM) analysis after Annexin V/PI staining. (A) The representative figures of cells with different treatments detected by FCM analysis; (B) Increase of cell apoptosis rate induced by mtg_iRNA1, mtg_iRNA2 and mtg_iRNA3 ($P<0.05$).

response. The design could be as an alternative way to open its new applications in RNAi based gene therapy and drug development.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL and YYZ designed the study. YYZ designed the different structures of multi-target interfering RNAs. TL, YJ and SZ performed the RT-qPCR, western blot analysis, MTT. TL performed the cell invasion assay and cell apoptosis assay. TL was a major contributor in writing the manuscript; YYZ revised the manuscript and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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