Downregulation of human choline kinase α gene expression by miR-876-5p

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Abstract. Choline kinase (CK) is the first enzyme in the CDP-choline pathway for the synthesis of phosphatidylcholine, the most abundant phospholipid in the mammalian cell membrane. This enzyme exists as three isozymes (α1, α2 and β) and the CKα isozyme has been implicated in cancer pathogenesis. Inhibition of CK activity has been proposed for cancer therapies. MicroRNAs (miRNAs/miRs) are non-coding RNAs that serve important roles in diverse biological pathways and human diseases, including cancer. However, the regulation of CKα gene expression by miRNAs has never been investigated, to the best of the authors' knowledge. In the present study, two miRNA mimics, miR-876-5p and miR-646, were transfected into the HepG2 cell line and the effect of these miRNAs on the levels of CKα mRNA were determined by reverse transcription-quantitative polymerase chain reaction. Cells transfected with 25 nM miR-876-5p for 48 h exhibited significantly lower levels of CKα mRNA. Following optimization, miR-876-5p caused four times lower levels of CKα mRNA compared to the negative control. Effects of the miRNAs on HepG2 cell viability and cellular morphology were additionally analyzed using an MTT cell viability assay and scanning electron microscopy, respectively. HepG2 cells that were transfected with the optimum concentration of miR-876-5p for the optimum duration exhibited 25% lower viability than negative control and signs of apoptosis in electron micrographs. The results suggested miR-876-5p as a potential miRNA modulator of CKα expression in the cells, and may be relevant for the design of more effective anticancer strategy targeting CK.

Introduction

Choline kinase (CK) catalyzes the phosphoryl transfer from ATP to choline, yielding phosphocholine. CK is the first enzyme in the CDP-choline pathway for the biosynthesis of phosphatidylcholine, the most abundant phospholipid in the eukaryotic cell membrane (1). In humans, CK is encoded by two separate genes, termed CHKA (HGNC no. 1937; referred to as CKα in the present study) and CHKB (HGNC no. 1938; referred to as CKβ in the present study), that produce the CKα1, CKα2 and CKβ isozymes (2). Functionally, CKα and CKβ catalyze the same activity, although they have specific roles in the physiology and development of an organism (3). Human tumor tissues usually exhibit increased CKα activity that leads to elevated levels of its product, phosphocholine (4,5). Overexpression of CKα has additionally been reported in a variety of types of human cancer including lung, colorectal and prostate adenocarcinomas (5-7). CK overexpression additionally increases the invasiveness and drug resistance of breast cancer cells (8). CKα inhibitors act as potent antitumor drugs *in vitro* and *in vivo*, as demonstrated by Rodríguez-González *et al* (9). Specific inhibition of CKα expression triggers apoptosis and selectively kills cancer cells, although not normal cells (10-12). Notably, the possible regulation of CK gene expression levels in the cells by miRNAs has never been examined, to the best of the authors' knowledge.

MicroRNAs (miRNAs) are small non-coding regulatory RNAs with a length of ~20 nucleotides that are involved in diverse cellular events, including cell development, neuronal cell fate, cell death and proliferation, and fat storage (13). MiRNAs achieve their functions by affecting gene expression through sequence-specific interactions with the 3'-untranslated regions (UTR) or 5'-UTR of target mRNAs which lead to translational repression and/or destabilization of the target mRNA (14-17). Dysregulation of miRNA expression is common in numerous types of cancer (18). In terms of drug discovery, strategies proposed for miRNA therapeutics include the artificial introduction of antisense oligonucleotides to inhibit miRNAs (19) and the design of an anti-miRNA oligonucleotide that is able to specifically inhibit the action of miRNAs (20). In addition, a novel hydrogel technology delivery of an antagomiR to inhibit an miRNA that promotes tumor growth in mice has been reported to effectively kill cancer cells (21). Furthermore, the reintroduction of tumor-suppressive miRNAs has been employed in order to block the functions of oncogenes (22). The majority of efforts have been directed at the inhibition of the activity of CKα by using small molecule inhibitors or RNA interference (RNAi), and little attention has been given to the regulation of CK expression by intracellular modulators,

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including miRNAs. Therefore, it may be useful to search for miRNAs that target CKα to discover their potential application in the treatment of certain types of cancer.

In the present study, two miRNAs, miR-876-5p and miR-646, predicted to target the CKα gene were studied for their potential regulation of CKα expression in HepG2 cells. The present study aimed to test experimentally the effect of miR-876-5p and miR-646 mimics on CKα gene expression in HepG2 cells. The results obtained in the present study reveal the involvement of miRNAs in modulating the expression level of CKα in cancer cells, something that may be developed into a promising anticancer therapeutic for the treatment of CKα-overexpressing cancers.

Materials and methods

Cell culture. The HepG2 cell line was obtained from the American Type Culture Collection (cat.no.HB-8065; Manassas, VA, USA) and maintained at 37˚C, 95% humidity and 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. The HepG2 cell line is a hepatoblastoma cell line previously misidentified as hepatocellular carcinoma (23). The HepG2 cells were seeded in a 24-well plate at a concentration of $1.5x10^5$ cells/well (diluted with antibiotic-free complete medium) 1 day prior to transfection with miRNAs.

Prediction of CKα-targeting miRNAs. TargetScan (www.targetscan.org), DIANA micro T (www.diana.imis.athena-innovation.gr), microRNA.org (www.microrna.org) and RepTar (reptar.ekmd.huji.ac.il) were used to predict the miRNAs that potentially bind to the 3'-UTR of CKα mRNA, using the default settings of the respective programs. The minimum free energy of the predicted miRNAs was calculated using RNAfold (rna.tbi.univie.ac.at), mFold (unafold.rna.albany.edu) and KineFold (kinefold.curie.fr). The site features of the mRNA-miRNA pairings in terms of G:C, A:U and G:U matches, mismatches and bulges in miRNAs and mRNAs were analyzed manually to search for high complementarity at the seed region and the minimum number of bulges. The potential miRNAs were additionally analyzed based on the classification of miRNA target sites, according to Brennecke *et al* (24), types of matching to seed region, according to Lewis *et al* (25) and site contexts, based on Grimson *et al* (26).

miRNA transfection. Non-targeting (miRIDIAN microRNA Mimic Negative Control #1; GE Healthcare Dharmacon, Inc., Lafayette, CO, USA), GAPDH-targeting (Mimic Housekeeping Positive Control #2; GE Healthcare Dharmacon, Inc.), miR-876-5p (MISSION® MicroRNA mimic HMI0929, 5'-UGGAUUUCUUUGUGAAUCACCA-3'; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) and miR-646 (MISSION® MicroRNA mimic HMI0877, 5'-AAGCAGCUG CCUCUGAGGC-3'; Sigma Aldrich; Merck KGaA) miRNAs were re-suspended in 1X miRNA buffer (GE Healthcare Dharmacon, Inc.) to prepare a stock solution of 20 μM. The non-targeting (negative control) miRNA sequence was based on the *Caenorhabditis elegans* cel-miR-67 that was confirmed to exhibit minimal sequence identity with miRNAs from humans, mice and rats. The GAPDH-targeting miRNA (referred to as miRNA-GAPDH) targets the 3'UTR of GAPDH mRNA. The miRNA stock solution was further diluted with RNase-free water to obtain a 2 μM working solution. Prior to the transfection, the miRNAs were diluted with serum free Opti-Minimum Essential Medium (MEM)® I (Thermo Fisher Scientific, Inc.), such that the final concentrations in the treatment plate were 25, 50 and 100 nM. In a separate tube, DharmaFECT 2 transfection reagent (GE Healthcare Life Sciences, Little Chalfont, UK) was additionally diluted with the serum free Opti-MEM®, according to the manufacturer's protocol. The diluted miRNAs and transfection reagent were mixed for 20 min at room temperature to form transfection complexes. The culture medium from the 24-well plate was removed and 400 μl complete medium plus 100 μl transfection complexes were added into each well and cultured at 37˚C with 5% CO_2, for a duration of 24 to 48 h. For screening of miRNA mimics for the downregulation of CKα mRNA levels in the HepG2 cancer cell line, the cells were transfected for 48 h.

Extraction of total cellular RNA. Following the transfection, the medium was removed and the cells were trypsinized, harvested and washed with PBS prior to RNA extraction using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Any residual DNA was eliminated by adding RNase-Free DNase I (Qiagen GmbH) onto the spin column and incubating for 20 min at room temperature. The concentration of the purified total RNA was measured at 260 nm and the $OD_{260/280}$ was determined using a BioPhotometer Plus (Eppendorf, Hamburg, Germany). The RNA integrity and size distribution were assessed by running the purified RNA samples on a 1% agarose gel.

Synthesis of cDNA. The first strand cDNA was synthesized from the extracted RNA using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) with a MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 1 μg total RNA was mixed with 0.5 μl each of oligo$(dT)_{18}$ (50 μM) and random hexamers (50 μM), with the final volume made up to 12 μl with distilled water. The mixture was incubated at 65˚C for 5 min. Subsequently, 4 μl 5X reaction buffer, 1 μl RiboLock™ RNase Inhibitor (20 U/μl), 2 μl dNTP mix (10 mM) and 1 μl RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 U/μl) was added into the mixture, and incubated at 25˚C for 5 min and 42˚C for 1 h; the reaction was terminated by a final incubation at 70˚C for 5 min. The synthesized cDNA was stored at -20˚C until use.

Quantitative polymerase chain reaction (qPCR). The qPCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reactions were performed in 96-well plates (Axygen; Corning Incorporated, Corning, NY, USA). A relative quantification method with ubiquitin C (UBC) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ (YWHAZ) as reference genes was used. A negative control without template DNA was run together with the other samples to detect possible contamination or

non-specific amplification in the reaction. The PCR reactions with a final volume of 25 μl consisted of 12.5 μl Power SYBR-Green I Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 1.5 μl gene specific primers, 1 μl 1:2 diluted cDNA and water. The cycling parameters were 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 10 sec at 95˚C and 1 min at 60˚C. Following the amplification, PCR specificity was verified by melting curve analysis with temperatures ranging between 60 and 95˚C with 0.1˚C increments. All reactions were run in triplicate. The reference genes (UBC and YWHAZ) were selected based on a previous study (27). The forward primer sequence for UBC was 5'-CTGATCAGCAGAGGTTGATCTT-3' and its reverse primer sequence was 5'-GTCTTGCCAGTGAGTGTCTT-3'. For YWHAZ, the forward primer sequence was 5'-TTCTTG ATCCCCAATGCTTC-3' and the reverse primer sequence was 5'-AGTTAAGGGCCAGACCCAGT-3'. The forward and reverse primer sequences for GAPDH were 5'-CAAGGTCAT CCATGACAACTTTG-3' and 5'-GTCCACCACCCTGTT GCTGTAG-3', respectively. The forward primer sequence for total CKα was 5'-TCAGAGCAAACATCCGGAAGT-3' and the reverse primer sequence for total CKα was 5'-GGCGTA GTCCATGTACCCAAAT-3'. The forward and reverse primer sequences for CKα2 specific amplification were 5'-GGCCTT AGCAACATGCTGTTC-3' and 5'-AGCTTGTTCAGAGCC CTCTTT-3', respectively. Relative gene expression levels normalized to the geometric mean of UBC and YWHAZ C_q values were determined by the $2^{-\Delta\Delta Cq}$ method (28).

MTT cell viability assay. The MTT cell viability assay was performed by seeding $1.5x10^4$ HepG2 cells in 100 μl antibiotic-free complete medium into each well of a 96-well plate and incubating at 37˚C with 5% CO_2 1 day prior to the transfection with miR-876-5p mimic. The cells were transfected with 25 nM miR-876-5p for 48 h with a final volume of 100 μl in each well. The MTT assay was performed by replacing the medium following transfection with 100 μl fresh culture medium, followed by the addition of 10 μl 12 mM MTT stock solution and incubation at 37˚C for 4 h. Following that, 85 μl medium was removed and 50 μl dimethyl sulfoxide (DMSO) as the solubilizing agent to dissolve the formazan dye was added to each well and thoroughly mixed by pipetting, prior to further incubation at 37˚C for 10 min. Finally, the absorbance at 540 nm was read by using microplate reader (Bio-Rad Laboratories, Inc.) and the percentage cell viability was calculated based on the average absorbance values for the samples and control.

Scanning electron microscopy. The morphological alterations in HepG2 cells following treatment with either DMSO or the desired miRNAs for 48 h were observed under a scanning electron microscope (Quanta 200; FEI; Thermo Fisher Scientific, Inc.). Following transfection, cells were grown on a plastic cover slip (Thermo Fisher Scientific, Inc.) and were fixed with McDowell-Trump fixative solution [1% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M phosphate buffer; pH 7.2] (29) at 4˚C for 24 h. The following day, the samples were washed with 0.1 M PBS and incubated in 1% osmium tetroxide at room temperature for 1-2 h for secondary fixation. The samples were dehydrated via sequential incubation of 15 min each in 50, 75, 95 and 100% ethanol. The dehydrated samples were immersed in hexamethyldisilazane (HMDS): acetone (1:1 ratio) for 15 min and in 100% HMDS three times, for 15 min each, prior to being left overnight in a desiccator. The dried samples were mounted onto the sample stub prior to being coated with gold and viewed under the scanning electron microscope.

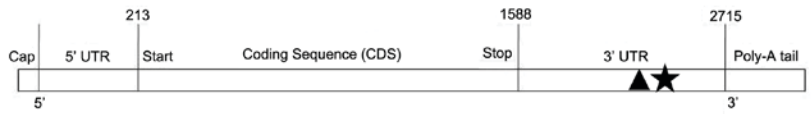
Statistical analysis. All data were analyzed using Student's t-test and one-way analysis of variance with the Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference, and all analyses were performed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). All data are presented as the mean ± standard error of mean from three independent experiments.

Results

Selection of miRNAs for downregulation of CKα gene expression. A total of 54 non-repeating miRNAs were predicted to target CKα mRNA by the online programs used in the present study. Of these, 22 miRNAs were shortlisted based on their sequence conservation among different species. Subsequently, the miRNAs were selected based on favorable minimum free energy (≤-1.0 kcal/mol), miRNA-mRNA binding site features at the seed region, complementarity (either the stronger 5'dominant or the weaker 3'compensatory), types of matching to the seed region (with site efficacy following this order: 8mer>7mer-m8>7mer-A1>6mer>Offset 6 mer) and other site contexts that improve efficacy (extra Watson-Crick base pairing at nucleotides 12-17 of the miRNA, and target sites that are located ≥15 nucleotides downstream of the stop codon, near either end of the 3'-UTR).

Based on the selection criteria described above, two miRNA candidates (miR-876-5p and miR-646) were selected for experimental validation. The principal features of these two miRNAs and the locations of their target sites at the 3'-UTR of CKα mRNA are presented in Fig. 1. In addition to exhibiting other favorable characteristics, miR-876-5p was selected based on its 8mer site type and two Watson-Crick base pairings at nucleotides 12-17. Although miR-646 only has a 7mer-m8 site type, it was selected for the four Watson-Crick base pairings at nucleotides 12-17 and the high GC base pairing throughout the whole miRNA sequence with its target site. In summary, the two miRNAs possess favorable seed types, pairings at nucleotides 12-17, distances from the stop codon, minimum free energy, and the absence of GU wobble pairs and mismatches at the seed region. The two miRNAs additionally possess the 5'dominant canonical target site type, which is more effective than the 5'dominant seed and 3'compensatory types.

Screening of miRNA mimics for the downregulation of CKα mRNA levels in the HepG2 cancer cell line. The levels of CKα mRNA were determined by reverse transcription-qPCR following transfection of HepG2 cells with 25 nM different miRNA mimics for 48 h. Fig. 2 illustrates that miR-876-5p mimic significantly ($P<0.05$) downregulated the expression of total CKα and CKα2 by ~0.8 and 0.7-fold, respectively. miR-646 did not significantly affect the expression levels of total CKα and CKα2 mRNA. The successful transfection



Features	miR-876-5p	miR-646
Matching at the seed region (bold) and Watson-Crick pairing at nucleotide 12-17 (highlighted)	mRNA 5'GAGUG--UAGCUGU**GAAAUCCA**3' 3'ACCACUAAGUGUUU**CUUUAGGU**5' miRNA	mRNA 5'-CC-CAGGGGAGCC**AGCUGCUU**3' 3'CGGAGUCUCC---G**UCGACGAA**5' miRNA
Target site type	5' dominant (canonical)	5' dominant (canonical)
Type of matching	8mer	7mer-m8
Total Watson-Crick base pairing	13	16
Distance from stop codon	> 15 nucleotide away	> 15 nucleotide away
Minimum free energy	−1.20 kcal/mol	−3.40 kcal/mol

Figure 1. Target site locations at the CKα 3'-UTR and principal features of miR-876-5p and miR-646. miR-876-5p (★) and miR-646 (▲) match with nucleotides 2562-2581 and 2355-2374 of the CKα mRNA transcript (no. NM_001277.2). miR/miRNA, microRNA; CKα, choline kinase α; UTR, untranslated region.

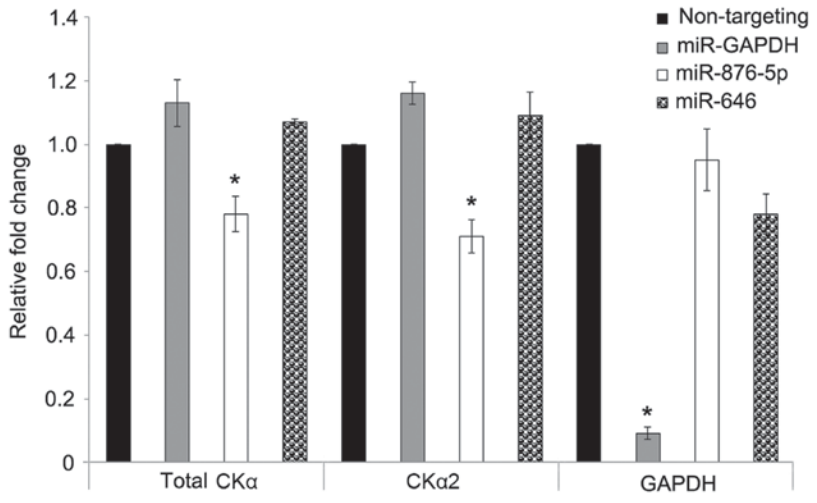


Figure 2. Effect of different miRNA mimic transfections on the expression levels of CKα mRNA in the HepG2 cell line. The cells were transfected with 25 nM miRNA for 48 h and the relative levels of CKα mRNA were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. non-targeting negative control. miR/miRNA, microRNA; CKα, choline kinase α.

of HepG2 cells with the miRNAs was confirmed by the GAPDH-targeting miRNA (positive control miRNA), which resulted in strong downregulation of GAPDH to ~10% compared with the negative control (90% downregulation). The results additionally demonstrated that CKα2 was the predominant CKα isoform, since the levels of downregulation

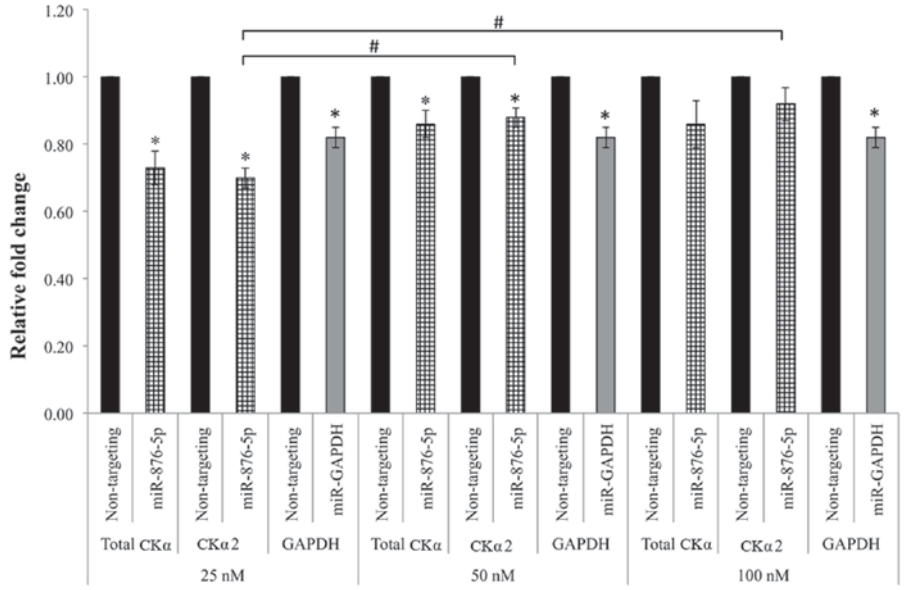


Figure 3. Effect of miR-876-5p mimic transfection concentration on the mRNA expression level of CKα. The relative fold changes of total CKα and CKα2 mRNA expression levels were determined following transfection of HepG2 cells with 25, 50 and 100 nM of miR-876-5p for 48 h. The non-targeting (negative control) and GAPDH-targeting (positive control) miRNAs were transfected at a concentration of 25 nM for 48 h. Error bars represent the standard error of the mean from three independent experiments. *P<0.05 vs. non-targeting negative control; #P<0.05. miR/miRNA, microRNA; CKα, choline kinase α.

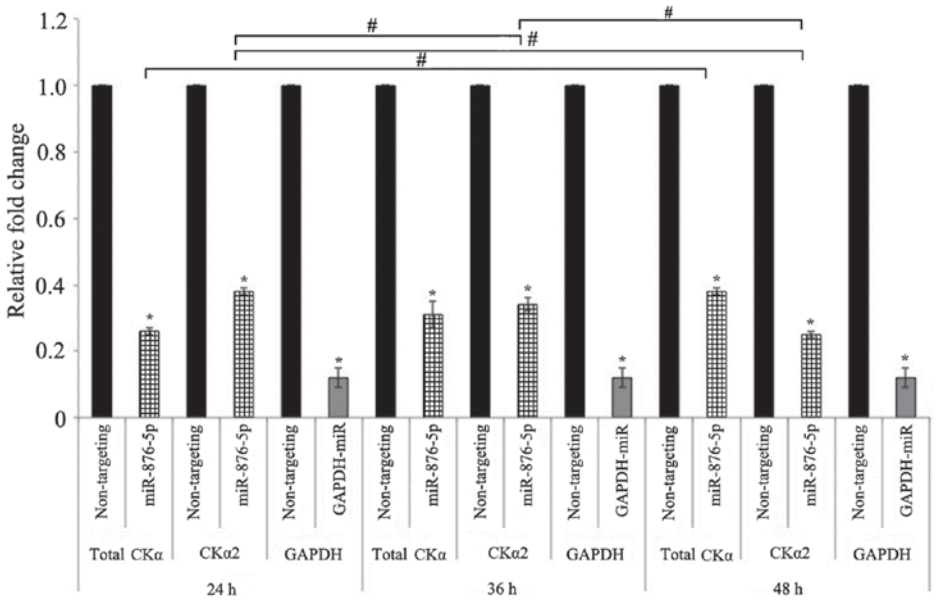


Figure 4. Effect of miR-876-5p mimics transfection duration on the mRNA expression level of CKα. The relative fold changes of total CKα and CKα2 mRNA expression levels were determined following transfection of HepG2 cells with 25 nM miR-876-5p for 24, 36 and 48 h. The non-targeting (negative control) and GAPDH-targeting (positive control) miRNAs were transfected at a concentration of 25 nM for 48 h. Error bars represent the standard error of the mean from three independent experiments. *P<0.05 vs. non-targeting negative control; #P<0.05. miR/miRNA, microRNA; CKα, choline kinase α.

by miR-876-5p for total CKα and CKα2 were very similar. Due to the significant downregulation of CKα mRNA by miR-876-5p, the effects of transfection concentration and duration were subsequently determined in the present study.

Effect of miR-876-5p concentration on the downregulation of CKα mRNA expression. A total of three concentrations of miR-876-5p were tested at the 48 h transfection duration. According to the results presented in Fig. 3, the total CKα and CKα2 mRNA expression levels were significantly downregulated to ~0.7 fold (P<0.05) compared with the negative control when the cells were transfected with 25 nM miR-876-5p. The total CKα and CKα2 mRNA expression levels were significantly downregulated (P<0.05) to 86% and 88%, respectively, compared with the negative control following the transfection with 50 nM miR-876-5p mimic. Transfection with 100 nM miR-876-5p mimic did not significantly downregulate the total CKα and CKα2 mRNA expression levels compared with the negative control (P>0.05). Comparison between the three concentrations demonstrated that there were significant

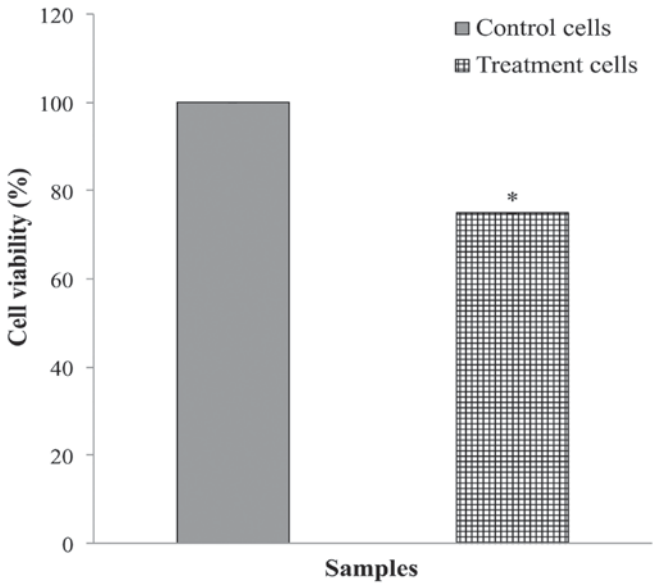


Figure 5. Effect of miRNA transfection on the viability of HepG2 cells. Cells were transfected with 25 nM non-targeting (negative control) and miR-876-5p miRNA mimic for 48 h. Cell viability was determined by MTT assay and expressed as a percentage relative to the negative control cells. *P<0.05 vs. control. miR/miRNA, microRNA.

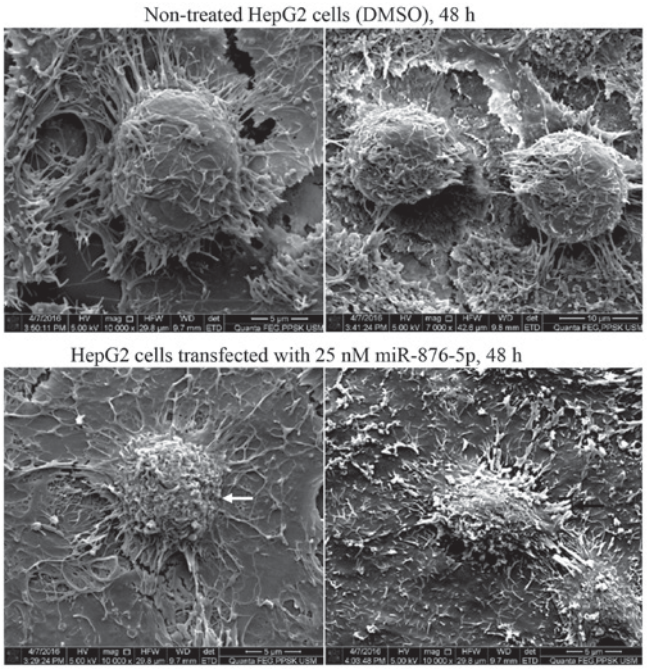


Figure 6. Effect of miR-876-5p transfection on the morphology of HepG2 cells. HepG2 cells were treated with either DMSO (negative control, upper panels) or 25 nM miR-876-5p miRNA mimic (lower panels) for 48 h prior to being processed and observed under scanning electron microscope, with a magnification of either x7,000 or x10,000. The arrowhead indicates cell blebs protruded from the plasma membrane. miR/miRNA, microRNA; DMSO, dimethyl sulfoxide.

differences between CKα2 levels in cells treated with 25 and 50 nM, and 25 and 100 nM (P<0.05). Notably, the downregulation caused by miR-876-5p may be underestimated for all the concentrations tested, as the level of GAPDH mRNA was not effectively downregulated by the positive control miRNA as in previous experiments. However, the overall results demonstrated that the concentration of 25 nM miR-876-5p mimic was able to produce the most marked downregulation of CKα gene expression.

Effect of miR-876-5p transfection duration on the downregulation of CKα mRNA expression. HepG2 cells were transfected with 25 nM miR-876-5p mimic at three different transfection durations. Fig. 4 illustrates that the expression levels of total CKα mRNA were significantly downregulated to 26, 31 and 38% compared with the negative control following transfection for 24, 36 and 48 h, respectively. The expression levels of CKα2 mRNA were additionally downregulated to 38, 34 and 25% compared with the negative control at the 24, 36 and 48 h transfection durations, respectively. All the downregulations were significant when compared with the negative control (P<0.05). Comparison between the three transfection durations demonstrated that there were significant differences in CKα2 expression levels between all the durations tested, while total CKα expression levels were only significantly different between cells transfected for 24 and 48 h (P<0.05). The results were more reliable compared with the experiments for the optimization of miR-876-5p concentration, as the GAPDH mRNA levels were markedly downregulated by the positive control miRNA at all transfection durations tested. Since human CKα2 is more active than the CKα1 isoform (11) and the strongest downregulation of CKα2 mRNA expression levels in the HepG2 cell line was obtained with 48 h transfection of 25 nM miR-876-5p mimic, these parameters were selected for subsequent experiments. Taken together, the results affirm the ability of miR-876-5p to downregulate CKα gene expression.

Effect of miR-876-5p on HepG2 cell viability and cellular morphology. The viability of HepG2 cells following transfection with miR-876-5p was determined by MTT cell proliferation assay. As presented in Fig. 5, the viability of HepG2 cells transfected with 25 nM miR-876-5p mimic for 48 h was ~25% lower compared with cells transfected with the non-targeting miRNA. There was a significant difference (P<0.05) between the downregulation levels of miR-876-5p and the negative control miRNA.

Scanning electron microscopy was used to investigate the effect of miR-876-5p transfection on HepG2 cellular morphology, particularly the effect on the cell membrane structure. Fig. 6 presents representative images of non-treated and miR-876-5p-treated HepG2 cells. The majority of the non-treated cells (upper panels) appeared healthy, exhibiting a round shape with filopodia structures at the edges of their surfaces. By contrast, the cells transfected with miR-876-5p exhibited signs of apoptosis, including cell shrinkage, membrane blebbing and loss of filopodia on their membranes. Since the non-treated cells were not exposed to the liposomal transfection reagent, it must be noted that the apoptosis-indicating morphology observed in miR-876-5p-transfected cells may be due to the transfection reagent.

Discussion

Gene expression may be negatively regulated by non-coding, single-stranded RNAs of ~22 nucleotides, termed miRNAs,

which cause mRNA cleavage or translational repression (30). In the present study, miRNAs that potentially bind to the 3'-UTR of CKα mRNA were predicted by four online programs that used dissimilar algorithms and approaches. TargetScan, RepTar, microRNA.org and DIANA micro T flexibly allow users to search for potential miRNAs, according to target gene name (31). A total of eight features were taken into consideration for selecting the two best miRNAs: i) Minimum free energy; ii) mismatches at the seed region; iii) G:U wobble pair; iv) miRNA target site; v) miRNA site type; vi) Watson-Crick pairing at nucleotides 12-17; vii) distance from stop codon; and viii) its position at the 3' UTR of mRNA.

Human hsa-miR-876-5p (MiRBase accession no. MIMAT0004924, http://www.mirbase.org) with the sequence 5'-UGGAUUUCUUUGUGAAUCACCA-3' originates from the stem-loop (MiRBase accession no. MI0005542) encoded by a microRNA gene (NCBI accession no. NR_030597), located on chromosome 9. Recently, the serum level of miR-876-5p has been identified to be elevated by 9.5 fold in response to severe human enterovirus 71 infections (32). Knockdown of miR-876-5p reduced viral load in cultured cells and rendered EV71-related symptoms less severe in infected mice (32). It has been reported that miR-876-5p is expressed in primary human myocytes, and that oxytocin may modulate the expression of this miRNA (33). miR-876-5p appears to be one of the most downregulated miRNAs in lymph nodes metastases (34), while higher expression of miR-876-5p is correlated with longer overall survival rates of hepatocellular carcinoma (35), rendering this miRNA a potential target for liver cancer therapy (36). Liu *et al* (35) demonstrated that miR-876-5p was one of the top eight miRNAs targeting nima related kinase 11 for downregulation, leading to drug resistance in ovarian cancer. Human hsa-miR-646 (MiRBase accession no. MIMAT0003316) with the sequence 5'-AAGCAGCUGCCUCUGAGGC-3' originates from the stem-loop (MiRBase accession no. MI0003661) and is encoded by a microRNA gene (NCBI accession no. NR_030376) located on chromosome 20. miR-646 was initially recognized in a study of miRNAs expressed in human cerebral cortical gray and white matter (37). It was reported to be downregulated in numerous types of cancer, and to be of importance as a tumor suppressor (38). Accelerated osteosarcoma tumor progression according to Tumor, Node, Metastasis classification, and a large tumor diameter were the outcomes of decreased expression of miR-646 (38). Lower expression of miR-646 was associated with the metastasis of osteosarcoma, and it was identified that this miRNA downregulated the expression of fibroblast growth factor 2 to inhibit osteosarcoma metastasis (37). miR-646 additionally downregulated nin one binding protein, and inhibited the growth and proliferation of renal cancer cells. Downregulation of this miRNA resulted in the metastasis of renal carcinoma (39). However, the correlation between miR-876-5p and miR-646, and human choline kinase gene expression, is yet to be reported, to the best of the authors' knowledge.

In the present study, the use of as low as 25 nM miRNA was sufficient to downregulate the expression of the target gene. This concentration is acceptable as it has been reported that miRNA concentrations >100 nM may decrease cell viability due to apparent toxicity (40). Higher concentrations may cause off-target or nonspecific effects, as demonstrated by Borawski *et al* (41). At a very low concentration of miRNA, the target gene expression may not be efficiently repressed and subtle downregulation of target gene expression may be difficult to measure (42).

The transfection duration requires optimization in order to determine the time point following transfection where target gene downregulation is the strongest. A prolonged transfection duration may affect cell survival and may not be suitable. A significant downregulation of the target gene mRNA expression level may be readily observed at 24 h post-transfection and the effect may become stronger with longer transfection duration, with the suppression of protein expression usually detected at 12 to 24 h following mRNA downregulation, depending on the half-life of the protein (43).

Total CKα mRNA was downregulated to ~26% of the negative control with 25 nM miR-876-5p at 24 h, while CKα2 mRNA was downregulated to ~25% of the negative control with the same amount of miR-876-5p at 48 h post-transfection. The magnitude of CKα mRNA downregulation exhibited by miR-876-5p was relatively large since, according to Mukherji *et al* (44), typical miRNA gene repression is relatively small when measured in a whole population of cultured cells. The results of the present study support the use of bioinformatics prediction of miRNAs targeting a specific gene of interest as a promising approach to search for novel miRNA modulators of the target gene. The reason for miR-646 not exerting any significant downregulation of CKα is unclear. The effect of miR-646 may require other transfection conditions. However, it may be that miR-646 does not bind to the 3'-UTR of CKα mRNA, although it was one of the best predicted candidates. It must be emphasized that further experiments are required to confirm the downregulation of CKα by miR-876-5p and to address certain limitations of the present study. These limitations include the lack of proof of a direct interaction between miR-876-5p and the 3'-UTR of CKα mRNA, and this requires further investigation with a luciferase reporter assay. Western blot analysis is require to confirm the alterations in CKα protein expression levels following treatment with miR-876-5p. An additional limitation of the present study was the variation in transfection efficiency, as illustrated by the unusually low GAPDH downregulation in the experiments to determine the optimal miR-876-5p concentration compared to the results of the miRNA screening and determination of optimal miR-876-5p transfection duration. The experiments ought to be repeated to obtain more accurate results for the determination of the optimum miR-876-5p transfection concentration.

The results of the cell viability assay and scanning electron microscopy additionally suggested that miRNA-876-5p induced suppression of CKα gene expression, caused decreased cell viability and induced apoptotic cell death. However, further experiments are required to verify these results. Future studies may include cell proliferation and apoptosis assays at different time points, and the negative control cells may be transfected with non-targeting miRNA for direct comparison with miR-876-5p-transfected cells under the scanning electron microscope. Previously, knockdown of CKα expression by RNAi has been demonstrated to promote cancer cell death (11). Thus, miR-876-5p downregulation may generate a similar effect on cancer cells. The present study is the first, to the best

of our knowledge, to report on the miRNA regulation of CKα gene expression, and the miR-876-5p identified here may be developed into a promising anticancer therapeutic agent.

In conclusion, the present study demonstrated that, out of the two tested miRNA candidates, miR-876-5p was able to significantly decrease the expression level of CKα mRNA. At concentrations as low as 25 nM, miR-876-5p mimic was able to exert the strongest downregulation of CKα gene expression in HepG2 cells. Additionally, HepG2 cells transfected with miR-876-5p exhibited decreased viability compared with the non-treated cells, in addition to certain signs of apoptosis. All of these observations indicated the role of miR-876-5p in modulating CKα gene expression and its direct involvement in cancer cell proliferation. Further studies are required to examine the expression pattern of miR-876-5p in different tumors, and to investigate the association between the level of this miRNA with tumor type or invasiveness. The results of the present study may be further confirmed by looking at the effect of treating appropriate cell lines with anti-miR-876-5p oligonucleotides on CKα gene expression. In addition, it is also important to demonstrate that the effect observed in the present study was due to direct binding of miR-876-5p to the 5'-UTR of CKα mRNA by luciferase reporter assay. The effect of this miRNA on the CKα protein expression level requires investigation.

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Availability of data and materials

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

Authors' contributions

SMAK performed the experiments, analyzed the data and wrote the manuscript. LLF designed the experiments, analyzed the data and wrote the manuscript. WCST conceived and designed the experiments, analyzed the data and was the major contributor in writing the manuscript. 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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