MicroRNA-367-3p induces apoptosis and suppresses migration of MCF-7 cells by downregulating the expression of human choline kinase α

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Abstract. Choline kinase (ChK) catalyzes the first step in the CDP-choline pathway for the synthesis of phosphatidylcholine. The α isoform of this enzyme is overexpressed in various types of cancer and its inhibition or downregulation has been applied as an anticancer strategy. In spite of increasing attention being paid to ChK expression, as well as its activity and inhibition in cancer, there are only limited studies available on the regulation of ChK, including its regulation by microRNAs (miRNAs/miRs). The dysregulation of gene expression by miRNAs is a common cause for carcinogenesis. In the present study, miR-367-3p was predicted to target the 3'-untranslated region (UTR) of the ChK α (chka) mRNA transcript. The binding of miR-367-3p to the 3'-UTR of chka was validated by a luciferase assay. The effects of the miR-367-3p mimic on chka gene and protein expression levels were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. miR-367-3p significantly downregulated the expression of chka to ~60% of the negative control. Cells transfected with miR-367-3p exhibited higher levels of apoptosis and a lower cell migration compared with the control. To the best of our knowledge, the present study provided the first experimental evidence of the regulation of chka expression by miR-367-3p. The pro-apoptotic and suppressive effects of miR-367-3p on cell migration were similar to the anticancer effects resulting from the inhibition of ChK enzyme activity or the knockdown of chka gene expression by small interfering RNA. Therefore, these findings may potentially lead to the use of miR-367-3p in anticancer strategies that target ChK.

Introduction

Choline kinase (ChK) catalyzes the phosphorylation of choline by using ATP as a phosphoryl donor. It is the first enzyme in the CDP-choline pathway for the de novo biosynthesis of phosphatidylcholine, the most abundant phospholipid in eukaryotic cell membrane (1). There are three isoforms of Chk in humans that are encoded by two separate genes known as chka and chkb. chkb codes for a single protein (ChKB), while chka undergoes alternative splicing to produce ChKA1 and ChKA2 isoforms (1). ChKA has been implicated in carcinogenesis, as this isoform is overexpressed in a variety of cancer types, including lung, breast, colon, ovarian and prostate cancer (2). By contrast, ChKB is crucial for muscle development, mitochondrial function and bone homeostasis (3).

ChKA serves an essential role in cell proliferation and transformation, as well as in the regulation of apoptosis and the cell cycle (4). The level of Chk and its product, phosphocholine are diagnostic indicators of cancer and markers for monitoring the tumor response to therapies (5). Due to the higher level of Chk expression in different cancer cells, the inhibition of ChKA activity has become a promising anticancer therapy. Consequently, the synthesis and testing of various ChKA inhibitors have gained increasing attention over the past decade (6). However, limited attention has been paid to the regulation of chk gene expression, particularly by microRNAs (miRNAs/miRs).

miRNAs are a large family of non-coding RNAs of ~21 nucleotides in length that regulate gene expression either by the degradation of target miRNAs or the inhibition of protein translation (7). miRNAs serve important roles in the immune system, differentiation, tumorigenesis and cell death (8). The dysregulation of miRNA expression is common in a number of types of cancer, and the miRNA profiling of clinical samples may be used for cancer diagnostic and prognostic applications (9). In humans, miR-195-5p functions as an anti-oncogene by targeting PHF-19, leading to the suppression of hepatoma cell invasion, migration and proliferation (10). Similarly, miR-7 has been demonstrated to exert an anti-metastatic effect on gastric cancer by targeting insulin-like growth factor-1 receptor (11). In certain cases, including the suppression of the Myc oncogenic pathway, the combined effects of several miRNAs have been observed (12). Therefore, a combined miRNA therapeutics approach to target lung cancer has been
proposed (13). In terms of drug discovery, the links between miRNAs and numerous human diseases and advances in anti-miR chemistries (chemical modifications for improved therapeutic properties) have suggested that the regulation of miRNAs may lead to the next revolution in pharmaceutical research (14). Until recently, there were ~20 clinical trials using miRNA- and small interfering RNA (siRNA)-based therapeutics (15). Strategies proposed for miRNA therapeutics include the artificial introduction of miRNAs or antisense oligonucleotides to inhibit miRNAs (16). In the present study, miR-367-3p was predicted and experimentally validated for the potential regulation of chka gene expression in breast cancer MCF-7 cells. The transfection of MCF-7 cells with miR-367-3p significantly downregulated chka expression, induced apoptosis and suppressed cell migration.

Materials and methods

Prediction of miRNAs targeting the human chka mRNA transcript. The prediction of miRNAs targeting the human chka mRNA transcript was performed using the microRNA.org website by submitting CHKA as the query gene symbol. The predicted miRNAs were ranked according to the mirSVR score (17). Minimum free energy (MFE) for miRNA binding to the target was predicted by RNAfold (18), mFold (19) and KineFold (20).

Cell lines, miRNA mimics, miRNA inhibitors, and chka-3'-untranslatedregion(UTR)fireflyluciferasereporterplasmid. The malignant breast cancer MCF-7 (ATCC® HTB-22™) cell line, the cervical cancer HeLa (ATCC®CCL2™) cell line and the hepatoblastoma HepG2 (ATCC®HB8065™) cell line were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; cat. no. 41965039, Thermo Fisher Scientific, Inc.), containing 10% heat-inactivated fetal bovine serum (FBS; Gibco; cat. no. 26140087, Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (cat. no. P0781, Sigma-Aldrich; Merck KGaA) and 100 mg/ml streptomycin (cat. no. 15140122, Sigma-Aldrich; Merck KGaA) in an incubator at 37°C and 5% CO₂. The mimic for miR-367-3p (5'-AAUUGGACUUUGGCAUUGGUGA-3') and its inhibitor were purchased from Applied Biological Materials (cat. no. MCH01999). miRIDIAN microRNA mimic Housekeeping Positive Control #2 (GAPDH) (cat. no. CP-001000-02-05) and miRIDIAN microRNA mimic Negative Control #1 (cat. no. CN-001000-01-05) were purchased from GE Healthcare Dharmacon, Inc. The miRNA mimic was reconstituted in 1X siRNA buffer provided by the manufacturer. The firefly luciferase reporter construct containing the 3'-UTR of the chka gene (pMirTarget-chka-3'-UTR) was purchased from OriGene Technologies, Inc. (cat. no. SC212759).

Cell transfection. The miRNA mimics, inhibitors and plasmids were transiently transfected into adherent MCF-7 cells cultured in either 96-well (for miRNA target validation) or 24-well plates (for other experiments) using Lipofectamine® 3000 (cat. no. L3000015, Invitrogen; Thermo Fisher Scientific, Inc.). To begin with, 1x10⁴ and 1x10⁵ cells were seeded into 96-well and 24-well plates, respectively, and cultured for 16-18 h to achieve 70-80% confluency prior to transfection. The plasmid, miRNA mimic and miRNA inhibitor were diluted accordingly with Opti-MEM® (cat. no. 31985062, Thermo Fisher Scientific, Inc.) to obtain final transfected concentrations of 200 ng pMirTarget-chka-3'-UTR or pMiRTarget empty vector, 25 nM miRNA mimic and 25 nM miRNA inhibitor. In another tube, Lipofectamine 3000 reagent (cat. no. L3000015, Invitrogen; Thermo Fisher Scientific, Inc.) was diluted with Opti-MEM® according to the manufacturer's protocol. The two mixtures were mixed at a 1:1 ratio for 20 min at room temperature for the formation of transfection complexes. Target validation in the 96-well plate, 50 µl the transfection complexes were added to each well containing 50 µl fresh complete DMEM. The medium was replaced after 6 h and the cells were grown for a further 24 h prior to performing the firefly luciferase activity assay. For other experiments in the 24-well plate, 100 µl transfection complexes were added to each well containing 400 µl fresh complete DMEM, following by incubation for a further 48 h.

Firefly luciferase assay. After transfection with the pMirTarget-chka-3'-UTR plasmid containing the 3'-UTR of the chka gene together with mimic for miR-367-3p (5'-AAUUGGACUUUGGCAUUGGUGA-3') or microRNA Negative Control #1 (GE Healthcare Dharmacon, cat. no. CN-001000-01-05) and miR-367-3p inhibitor (Applied Biological Materials, cat. no. MCH01999) as described above, a total of 25 µl medium per well was removed from the 96-well plate and 75 µl the Dual-Glo luciferase reagent (cat. no. E2920, Promega Corporation) was added, followed by incubation at room temperature for 10 min. The firefly luciferase activity (relative light unit) was determined using the GloMax 20/20 luminometer (Promega Corporation). All luciferase assays were performed in triplicate and the experiment was repeated ≥2 times. The firefly luciferase activity of miRNA/inhibitor was normalized with that of miR-NC.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the MCF-7, HeLa or HepG2 cells transfected with miR-367-3p, miRIDIAN (negative control) or miR-GAPDH (positive control) using the Total RNA Isolation kit (cat. no. K0732, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and genomic DNA removal was performed using the RNase-Free DNase set (cat. no. 79256, Qiagen GmbH). The RNA concentration was assessed using a Nano Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was reverse transcribed at 42°C for 1 h using the RevertAid™H minus First Strand cDNA Synthesis kit (cat. no. K1631, Thermo Fisher Scientific, Inc.) in a total volume of 20 µl. qPCR was performed using an ABI Prism 7500 Sequence Detection system (Thermo Fisher Scientific, Inc.) in a total reaction volume of 13 µl consisting of 6.25 µl Power SYBR®Green PCR Master Mix (cat. no. 4367659, Thermo Fisher Scientific, Inc.), 0.5 µl each of forward and reverse primers, 1 µl cDNA and 4.75 µl double-distilled water. The PCR was run with an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C (10 sec) and 60°C (1 min);
one cycle consisting of 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec was introduced to obtain the dissociation curve for the analysis of PCR specificity. The primers used were as follows: Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein (YWHAZ) forward, 5'-TTCTTGATCCCCAAATGCTTC-3' and reverse, 5'-ACTGGTGCGCCCTTAACCT-3'; ribosomal protein S18 (RPS18) forward, 5'-TGGTTGGTGAGGAAGAC-3' and reverse, 5'-CTCCAGTCGGCTCAAGGTTCT-3'; GAPDH forward, 5'-CAAGGTCACTCATGACAACTTTG-3' and reverse, 5'-GTCACCACCGCTGTGCTTAG-3'; and chka forward, 5'-TCAGAGCACAACATCCGGAAATGT-3' and reverse, 5'-GGCGTATGTCATGATCCAAAAT-3'. Relative gene expression levels normalized to the geometric mean of YWHAZ and RPS18 Cq values were determined using the 2^ΔΔCq method (21).

**Western blot analysis.** Following 72 h of transfection, MCF-7 cell lysates were prepared using ProteoJET™ mammalian cell lysis reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The protein concentration of the cell lysate was determined using Bradford assay reagent (cat. no. 5000006, Bio-Rad Laboratories, Inc.). Protein samples (30 µg) were loaded into the well of 12% SDS-PAGE. Following separation with 12% SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane. Protein samples (30 µg) were loaded into the well of 12% SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane. Protein samples (30 µg) were loaded into the well of 12% SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane. Protein samples (30 µg) were loaded into the well of 12% SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane.

**Scratch wound healing assay.** Following treatment with miRNA mimics as described earlier, a diameter wide scratch was gently created using a P20 pipette tip into near confluent MCF-7 cells grown in a 6-well plate. The growth medium containing serum with detached cells after scratching was then discarded and the cells were washed twice before 2 ml fresh serum-free medium were added to each well. Images at the specified time points were captured using a DINO EYE eye-piece camera (AnMo Electronics Corporation) and the distance between the two edges of the scratch was measured using ImageJ 1.49b software (National Institutes of Health).

**Statistical analysis.** Data were analyzed using a Student's t-test or one-way analysis of variance followed by a Tukey's HSD 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.). Data are presented as the mean ± standard error of mean from 3 independent experiments unless otherwise stated.

**Results**

**A apoptotic and dead cell count.** Cellular apoptosis was examined using the Muse™ Annexin V & Dead Cell Assay kit (cat. no. MCH100105, Merck Millipore) and analyzed using the Muse™ Cell Analyzer from EMD Millipore, according to the manufacturer's protocol. Transfected cells were detached by trypsinization, washed with PBS and resuspended in fresh DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS to at least 1x10^6 cells/ml. In one tube, 100 µl cell suspension was mixed with 100 µl Muse™ Annexin V & Dead Cell reagent, vortexed using a benchtop vortex mixer and incubated for 30 min at room temperature in the dark. Stained cells were counted using a Muse™ Cell Analyzer, according to the manufacturer's protocol.

**miR-367-3p targets the 3'-UTR of chka mRNA.** A total of 25 potential miRNAs targeting the 3'-UTR of the human chka mRNA transcript was predicted by microRNA.org. Hsa-miR-367-3p (miRBase accession no. MIMAT0000719) exhibited the highest mirSVR score of -1.0819. This miRNA targets the sequence from nucleotides 1804 to 1825 of the chka mRNA transcript (NM_001277.2). The analysis of MFE by
RNAfold, mFold and KineFold predicted the strong binding of miR-367-3p to the target with MFE values of -3.1, -3.0 and -5.2 kcal/mol, respectively. As shown in Fig. 1, the base pairing at the seed region of miR-367-3p with the target sequence fell into the most favorable category known as 8-mer, which contains perfect Watson-Crick base pairing at position 2-8 of the miRNAs and an adenine nucleotide across from position 1 of the miRNAs (22).

Validation of the predicted miR-367-3p was performed using firefly luciferase and the 3'-UTR of the chka gene fusion construct (pMirTarget-chka-3'-UTR) in MCF-7 cells. As shown in Fig. 2, miR-367-3p significantly downregulated the relative firefly luciferase activity by ~40%, compared with the negative control miRNA-treated cells (P=0.033). Co-treatment with mir-367-3p inhibitor reversed the effect of its target miRNA, confirming the specific interaction between this miRNA and this target 3'-UTR. Treatment with the inhibitor alone slightly increased (not significant, P=0.9715) the relative firefly luciferase activity compared with the negative control, possibly due to the inhibition of endogenous miR-367-3p.

miR-367-3p downregulation of chka mRNA transcript is targeted and downregulated by miR-367-3p. The mimic of miR-367-3p was subsequently transfected into MCF-7 cells to assess the potential of this miRNA to downregulate the expression of the chka gene. As shown in Fig. 3 (left panel), miR-367-3p significantly downregulated chka expression to ~60%, compared with the negative control, which was similar to the level of downregulation observed in the aforementioned luciferase assay. Western blot analysis (Fig. 3, right panel) also revealed a significantly lower Chka protein expression of ~50%, compared with the negative control. The transfection of miR-367-3p into HeLa and HepG2 cell lines resulted in the significant suppression of chka mRNA levels (Fig. 4), as was observed in the MCF-7 cells. Taken together, these results confirmed the prediction that the chka mRNA transcript is targeted and downregulated by miR-367-3p.

miR-367-3p downregulation of chka expression induces cell death and suppresses cell migration. The inhibition of ChK activity and the knockdown of chka gene expression by RNA interference (RNAi) have been reported to induce cancer
Figure 4. Downregulation of chka mRNA expression by miR-367-3p in the HeLa and HepG2 cell lines. Cells were transfected with 25 nM miR-367-3p mimic and the chka mRNA level was determined by reverse transcription-quantitative polymerase chain reaction at 48 h post-transfection. Error bars indicate the standard error of the mean from triplicate experiments. *P<0.05, compared with transfection with the miRIDIAN negative control. chka, choline kinase α; miR, microRNA.

Figure 5. miR-367-3p induces a higher level of MCF-7 cell apoptosis. Cells were transfected with 25 nM each miRNA mimic for 48 h, stained with Muse™ Annexin V and Dead Cell reagent and counted with a Muse™ Cell analyzer. *P<0.05, compared with transfection with the miRIDIAN negative control. miR, microRNA; chka, choline kinase α.
cell death (4). Therefore, the effect of miR-367-3p mimic on cellular apoptosis was investigated in the present study. Based on the results presented in Fig. 5, statistically significant differences were observed in the percentages of live cells and total apoptotic cells between the cells transfected with negative control miRNA and the miR-367-3p-transfected cells. These results suggested that miR-367-3p induced cell death by targeting chka. The inhibition of ChKa activity has been demonstrated to decrease the migration of tumor cells (23, 24). Therefore, the present study investigated whether the downregulation of chka expression by miR-367-3p was able to suppress MCF-7 cell migration. As shown in Fig. 6, miR-367-3p treatment significantly suppressed cell migration, with only ~60% closure of the scratch, compared with complete closure in the untreated and negative control cells after 72 h.

Discussion

ChK overexpression has been implicated in various types of cancer. The inhibition of the activity and the downregulation of the expression of this enzyme have been applied as promising anticancer strategies (6). However, the mechanisms that lead to the higher expression of ChK in cancer cells are not yet well understood. The present study investigated the possibility of the regulation of chka gene expression by miRNAs. miR-367-3p was predicted to target the 3'-UTR of the chka mRNA transcript with strong affinity, based on low values of mirSVR and MFEs. Target prediction analysis is crucial as the subsequent experimental validation of miRNAs is time-consuming and costly (25). mirSVR offers the optimal performance among other prediction tools when the ranking of the output is required (25). MFE of a miRNA-target interaction is one of the determinants of the silencing efficiency, as indicated by the improved silencing of the Arabidopsis MYB81 gene by miR-159 following mutation to incorporate the sequence for improved binding by miR-159 (26).

miR-367-3p originates from the 3'-end of the hairpin loop sequence of miR-367, which is expressed as the miR-302/367 cluster. This cluster of miRNAs is mainly expressed in embryonic stem cells and is able to reprogram somatic cells into pluripotency (27). miR-367-3p has been reported to enhance the efficacy of Sorafenib chemotheraphy to suppress hepatocellular carcinoma metastasis by enhancing the pathway involving the androgen receptor (28). However, the ectopic expression of miR-367-3p in non-small cell lung cancer cells has been demonstrated to promote cell proliferation and cell cycle progression, and inhibit apoptosis (29). According to Yan et al (30), the same miRNA may serve different roles in different types of cancer cell by acting as either an oncogene or tumor suppressor. The contradictory effects of miR-367 (whether this was miR-367-3p or miR-367-5p was not specified) on cancer cell growth and the response to drugs have been reported (31-35). The overexpression of miR-367 in paclitaxel-sensitive ovarian cancer cells has been demonstrated to further enhance the sensitivity to this drug (31). The overexpression of miR-367 has also been demonstrated to inhibit the migration and invasion of gastric cancer (32). By contrast, a higher expression of miR-367 has been found to be associated with an unfavorable prognosis of high-grade glioma (33), resected non-small cell lung cancer (34) and pancreatic ductal adenocarcinoma (35). Therefore, the effects of miR-367-3p on cancer cell survival may be cell type-dependent. The results of the present study suggested that this miRNA may be used as a novel therapeutic agent against breast cancer cells overexpressing ChK. The suppression of Runx2 by miR-135 and miR-203 has been shown to inhibit breast cancer cell migration in vitro, as well as tumor growth and metastasis in vivo (36). The results of wound healing assay in the present study also demonstrated that the suppression of chka by miR-367-3p inhibited MCF-7 breast cancer cell migration in vitro, which may produce the same anti-metastatic effect in vivo.

Due to the higher level of chka expression in different cancer cells, the inhibition of Chka activity has become a promising anticancer therapy. Consequently, the synthesis and testing of various small-molecule Chka inhibitors have gained increasing attention over the past decade (6). The inhibition of Chka enzyme activity
or the knockdown of chka gene expression by RNAi specifically induces the death of cancer cells, but not that of normal cells (1). The RNAi of chka has been demonstrated to decrease tumor cell viability and enhance the efficacy of prodrug enzyme activation treatment in cancer therapy (37). Recently, degradable dextran nanopolymer, which is less toxic and more suitable for cancer therapy, has been synthesized to deliver siRNA targeting chka in breast cancer cells (38). mir-367-3p, which downregulates the chka gene, as reported in the present study, may also be used in a similar manner. Replenishing tumor suppressor miRNAs, including miR-34 by lipid nanoparticles, miR-200 by liposomal carriers and miR-26a by the adeno-associated virus-mediated expression is one the approaches in miRNA therapeutics (39). The downregulation of the chka gene by RNAi or miRNAs may be more effective than activity inhibition by small molecules alone in killing cancer cells, based on the ChKA non-catalytic role in promoting cancer cell survival (40).

The anti-proliferative effects of miRNAs have previously been reported in humans. For example, miR-195-5p functions as an anti-oncogene by targeting PHF-19, leading to the suppression of hepatoma cell invasion, migration and proliferation (10). Similarly, miR-7 has been shown to exert an anti-metastatic effect on gastric cancer by targeting insulin-like growth factor-1 receptor (11). In the present study, it was confirmed that the 3′-UTR of the chka transcript was the target of miR-367-3p and the expression of chka was downregulated by this miRNA. The decreased level of chka resulted in higher levels of apoptosis and a lower migration of MCF-7 cells. Although the expression of miR-367-3p was not determined following the transfection of its mimic or inhibitor, the downregulation of the (chka) gene expression by the miR-367-3p mimic was consistent throughout all the experiments in the present study. The results of the present study suggested that miR-367-3p may be a promising miRNA candidate for further investigation into the roles of miRNAs in cancer development involving the dysregulation of chka gene expression. However, further studies are required to elucidate the effects of chka downregulation by miR-367-3p on cell proliferation, invasion and anticancer drug resistance in more cancer cell lines in order to fully understand the role of chka attenuation by this miRNA in cancer.

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Availability of data and materials

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

Authors’ contributions

SR and SAFMS performed the experiments and analyzed the data. LLF designed the experiments and drafted the initial manuscript. WCST conceived and designed the experiments, analyzed the data and was a major contributor in drafting the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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