RESEARCH ARTICLE

Cinobufacin Suppresses Cell Proliferation via miR-494 in BGC-823 Gastric Cancer Cells

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Abstract

Cinobufacin is used clinically to treat patients with many solid malignant tumors. However, the mechanisms underlying action remain to be detailed. Our study focused on miRNAs involved in cinobufacin inhibition of GC cell proliferation. miRNA microarray analysis and real time PCR identified miR-494 as a significant cinobufacin-associated miRNA. *In vivo*, ectopic expression of miR-494 inhibited the proliferation and induced apoptosis of BGC-823 cells on CCK-8 and flow cytometry analysis. Further study verified BAG-1 (anti-apoptosis gene) to bea target of miR-494 by luciferase reporter assay and Western blotting. In summary, our study demonstrated that cinobufacin may inhibit the proliferation and promote the apoptosis of BGC-823 cells. Cinobufacin-associated miR-494 may indirectly be involved in cell proliferation and apoptosis by targeting BAG-1, pointing to use as a potential molecular target of cinobufacin in gastric cancer therapy.

Keywords: Cinobufacin - miR-494 - BAG-1 - gastric cancer - proliferation

Asian Pac J Cancer Prev, **15 (3)**, 1241-1245

Introduction

Gastric cancer (GC) is the fourth most prevalent malignant cancer worldwide and is the second most frequent cause of cancer death (Kamangar et al., 2006). Despite many advances made in GC therapy, the prognosis for patients with GC remains unsatisfying. Chemotherapy play a major role in the treatment of gastric cancer over the past twenty years, However, the project of chemotherapy, consist of 5-FU and cisplatinum, showed less validation and security. Therefore, it is urgent to search a new therapeutic agents and targeted novels for providing more clinical benefits and better outcomes in gastric therapy.

MicroRNAs are non-coding regulator RNAs of 21 to 25 nucleotides which regulate most of basal progress such as cell proliferation, survival, apoptosis, and differentiation by triggering either translational repression or mRNA degradation (Bartel et al., 2004). Many evidence showed that the expression of miRNAs altered in many kinds of cancers, which triggered a new illumination in the diagnosis and therapy of cancer (Esquela-Kerscher et al., 2006; Sassen et al., 2008). Emerging researches found that aberrant miRNAs could be important in tumorigenesis as oncogenes and tumor suppressor genes in GC (Du et al., 2009; DingL et al., 2010; Guo et al., 2010; Tie et al., 2010; Wang et al., 2010; Guo et al., 2011).

Cinobufacin, the dried secretion from the skin glands of Bufo, has been used clinically for over amillenium as a TCM to treat the patients with many solid malignant tumors such as liver, lung, pancreatic, and colorectal cancers in China (Qi et al., 2010). Many studies demonstrated that cinobufacin may inhibit cancer development by the mechanism of suppressing the tumor cells proliferation and inducing the tumor cells apoptosis. However, the research about the miRNA associated with cinobufacin in inhibiting the Gastric caner was less reported.

In our previous studies, we have demonstrated that cinobufacin could inhibit the cell proliferation rate of GC BGC-823 cells in a time- and dose-dependent manner by MTT assay.Therefore, in this study, we elucidated that cinobufacin could induce the expression of miR-494 in inhibiting the Gastric cancer cells. In addition, we revealed the contribution of miR-494 to the molecular mechanism involved in cinobufacin-induced suppressing proliferation in BGC-823 cells.

Materials and Methods

Cell culture and reagents

Human gastric cancer cell line BGC-823 was purchased from the Cell Bank of Shang Hai. Cells were routinely cultured with RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37°C in a humidified atmosphere with 5 % CO_2. Cinobufacin was purchased from Jinchan Corporation, Anhui Province, China, and was prepared with dimethyl sulfoxide (DMSO) at the concentration of 50 mg/ml stored as small aliquots at -20°C, and thawed and diluted as needed in cell culture medium.

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Table 1. Primer sequence of miR-494 and U6

Gene	Sequence	Size
U6		
F primer	ATTGGAACGATACAGAGAAGATT	70bp
R primer	GGAACGCTTCACGAATTTG	
hsa-miR-494		
F primer	TGACCTGAAACATACACGGGA	76bp
R primer	TATCGTTGTACTCCACTCCTTGAC	

MiRNA microarray analysis.

Prior to experimentation, cinobufacin-treated and untreated cells were analyzed by miRNA microarray. Total RNA was harvested using TRIzol (Invitrogen) and an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. After RNA quan¬tification using a Nanodrop spectrophotometer, the samples were labeled using the miRCURYHy3/Hy5 Power Labeling Kit and hybridized to the miRCURYLNAArray (v. 11.0). The samples were hybridized using a hybridization station and the arrays were scanned with the Axon GenePix 4000B Microarray Scanner. The raw intensity of the image was read using GenePix Pro V6.0. The intensity of the green signal was calculated after background subtraction, and four replicated spots for each probe on the same slide were averaged. The Median Normalization Method was used to obtain Normalized Data' [Normalized Data = (foreground-background)/median]. The median was defined as the 50% quantile of microRNA intensity that was >50 in all samples after background correc¬tion. The statistical significance of the differentially expressed miRNA was analyzed using the Student's t-test.

Real-time reverse transcriptase quantitative PCR

Total RNA was extracted from BGC-823 cells treated by cinobufacin with Trizol reagent (Invitrogen). The quality and quantity of the RNA samples were assessed by standard electrophoeresis and spectrophotometric methods. Real-time reverse transcriptase quantitative PCR (qRT-PCR) analysis were performed with locked nucleic acids (LNAs) linear primers (EXIQON) and SYBR Green I, and U6 small nuclear RNA was used as normalized PCR primers. All reagents for stem-loop RT and Q-PCR were obtained from TAKARA. The primers used for stem-loop RT-PCR and Q-PCR were synthesized and purified by RiboBio. The sequence of the primers was shown in Table 1. The PCR conditions were 95°C for 180s, followed by 40 cycles of 95°C for 12s, 62°Cfor 40s, 72°Cfor 30s. The reactions were monitored using a preheated real-time instrument (ABI step one). The relative expression ratio of miR-494 in BGC-823 cells was quantified by the $2^{-\triangle\triangle CT}$ method.

Transfection with miR-494 in constructs

To elevate miR-494 expression, a miR-494 mimic (GenePharma, Shanghai, China)was designed, a scrambled sequence was used as the Negative Control (nm) mock transfection were used as control (m). For transfection, cells were seeded in 6-well plates at a density of 1×10^5 cells/well and transfected with 20 μM miR-494 mimic, NC using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction.

Cell growth assay

BGC-823 cells were seeded in 96-well plates 1 day before transfection. Twenty-four hours after transfection, cells were trypsinized and seeded into 96-well culture plates at a density of 10, 000 cells/well in growth medium supplemented with 10 % serum. The cells were harvested at different time points (24, 48, 72, and 96 h) for growth assay. The miR-494 mimics (sense:5'-UGAAACAUACACGGGAAACCUC-3', antisense:5'-GGUUUCCCGUGUAUGUUUCAUU-3'), mimics control (sense:5'-UUCUCCGAACGUGUCACGUTT-3', antisense:ACGUGACACGUUCGGAGAATT-3'), and one day before transfection, 5.0 x 103 BGC cells in 100ul growth medium were plated in each well of a 96-well plate. The cells were then transfected with 50 nM of various synthetic miRNAs mimics and 100 nM inhibitor using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. Cell proliferation was assessed at different time points (0 h, 24 h, 48 h, and 72 h), using Cell Counting Kit 8 (Dojindo, Tokyo, Japan) according to manufacturer's protocol. Absorbance at a wave length of 450 nm, which shows positive relation to cellular proliferation, was measured by a spectrophotometer.

Flow cytometry analysis of apoptosis

Twenty-four hours after transfection, the cells were collected and washed with PBS twice and about 1×10^6 cells were resuspended in 400 μl Annexin V binding buffer (Mbchem M3036). The cells were stained with 5 μl Annexin V FITC (Mbchem M3031) for 15 min at 4°C in the dark. The reaction was stopped by the addition of 10 μl propidium iodide (PI) and incubation for 5 min at 4°C in the dark. Flow cytometry analysis was within 1 h.

Western blot analysis

Total cell lysates were prepared using RIPA buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.4, 1 % Triton X- 100, 0.5 % deoxycholic acid, 0.1 % SDS) and the Protein concentration was measured by BCA method. Equal amounts of proteins were separated by 10% SDS-PAGE and blotted to PVDF membranes (Millipore, USA). The membranes were blocked with 5 % non-fat milk powder at room temperature for 2h, then incubated with specific antibody for BAG-1 (ABGENT, USA) at 4°C overnight followed by incubation with secondary antibody (ABGENT, USA) for 1 h at room temperature. The membranes were developed using ECL kit (Pierce, USA) and exposed to X-ray film to visualize the images.

Luciferase reporter assay

miR-494 mimics or scramble lentiviruses (NC) and BAG-1-3'UTR vector were cotransfected into HEK-293T cells. Renilla and firefly luciferase activities were measured with the Dual-Luciferase Reporter system (Promega, Madison, WI) 24 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase expression for each sample. Each experiment was performed in triplicate.

Bioinformatic prediction for miR-494 targeted genes

TargetScan (http://www.targetscan.org), miRanda

Table 2. Up-regulations miRNAs and Down-regulations miRNAs of the miRNA Expression Profiles in BGC823 Cells Following Cinobufacin Treatment

Up-regulations folds miRNA		Down-regulations folds miRNA	
hsa-miR-454-3p	11.47	hsa-miR-183-5p	0.34
hsa-miR-29a-5p	4.48	hsa-miR-7-5p	0.40
hsa-miR-299-3p	3.78	hsa-miR-548as-3p	0.40
hsa-miR-301a-3p	2.87	hsa-miR-33b-5p	0.53
hsa-miR-335-5p	2.84	hsa-miR-1246	0.58
hsa-miR-26a-5p	2.66	hsa-miR-3613-3p	0.58
hsa-miR-107	2.65	hsa-miR-34a-5p	0.59
hsa-miR-493-5p	2.58	hsa-miR-32-3p	0.60
hsa-miR-494	2.51	hsa-miR-21-3p	0.61
hsa-miR-20b-5p	2.31	hsa-miR-1246	0.61
hsa-miR-29b-3p	2.29	hsa-miR-1246	0.63
hsa-miR-3178	2.28	hsa-miR-339-5p	0.64
hsa-miR-18a-5p	2.11		
hsa-miR-3651	2.02		
hsa-miR-21-5p	1.93		
hsa-miR-3177-3p	1.90		
hsa-miR-424-5p	1.89		
hsa-miR-4521	1.85		
hsa-miR-29a-3p	1.84		
hsa-miR-138-2-3p	1.77		
hsa-miR-27a-3p	1.75		
hsa-miR-19a-3p	1.72		
hsa-miR-24-1-5p	1.71		
hsa-miR-100-5p	1.71		
hsa-miR-15a-5p	1.66		
hsa-miR-5684	1.66		
hsa-miR-30d-5p	1.61		
hsa-miR-4500	1.59		
hsa-miR-4288	1.59		
hsa-miR-29a-3p	1.58		
hsa-miR-1273g-3p	1.52		
hsa-miR-4321	1.50		
hsa-miR-18b-5p	1.50		

(http://www.microrna.org) and PicTar (http://pictar.bio.nyu.edu/) online searching programs was used for the prediction of miR-494 target genes.

Statistical Analysis

Data were presented as mean ± SD, the significance was analyzed with the Student's t-Test, the statistical significance of correlation was calculated by chi-square test and Spearman's rank correlation. Statistical analysis was performed using SPSS 16.0 software and differences were considered statistically significant when $p< 0.05$.

Results

Effect of Cinobufacin on miRNA expression

After BGC-823 cell was treated with 50mg/ml of cinobufacin for 48h, changes in miRNA expression profiles in BGC-823 cells were shown by microarray data. Only the miRNAs that were upregulated by more than 1.5 folds or those down (- regulated by 66.7% compared to the control level, were considered as significantly differention. Based on data of miRNA microarray assay, 33 were upregulated and 12 were downregulated as shown in Table 2. Among which miR-494 was significantly upregulated. Therefore, miR-494 was chosen as a candidated miRNA to evaluate the role in the effect of Cinobufacin on the BGC-823 cells.

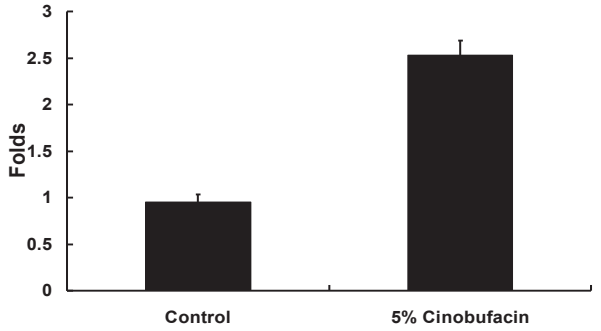

Figure 1. Relative mRNA Expression in Cells Exposed to Cinobufacin by qPCR

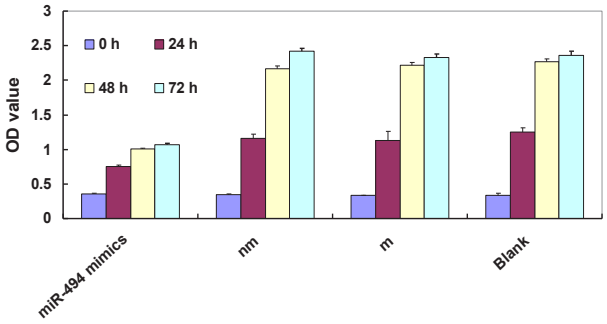

Figure 2. The OD Values of BGC823 Cells Exposed to miR-494 Mimics (nm:negative control; m: mock transfection control)

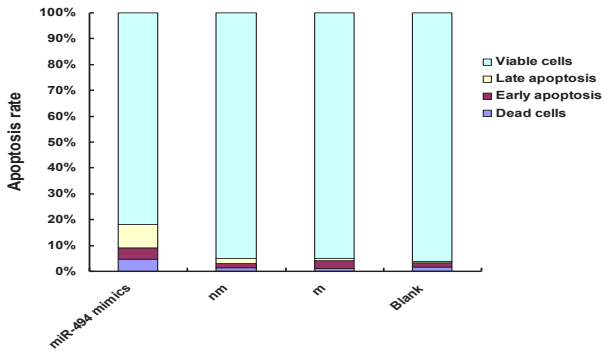

Figure 3. The Apoptosis Rates of BGC823 Cells Exposed to miR-494 Mimics (nm:negative control; m: mock transfection control)

miR-494 expression pattern and expression level was confirmed by qPCR

To further verify the miRNA array results, we performed qRT-PCR to measure the expression of miR-494. As is shown in Figure 1, the expression of miR-494 is consistent with the results of miRNA array results, suggesting that miRNA array results were reliable.

miR-494 inhibited the proliferation of BGC-823 cells

To assess the effect of miR-494 on the growth of gastric cancer cells, we transfected miR-494 mimic into BGC-823 cells. The OD value of cell growth revealed that miR-494 mimic inhibited cell proliferation compared with nm, m (Figure 2, *$p<0.05$).

miR-494 promotes the apoptosis of BGC-823 cells

Next, flow cytometry analysis was performed to examine apoptosis of BGC-823 cells transfected by miR-

Table 3. miR-494 Target Genes Predicted by Targetsan, miRanda, PicTar

BAG-1	BCL-10	CAPS2	CDH11	CD28	DNHD1	UACA	UFM1
DSC2	MTMR2	RBBP7	TAF5	FHAD1	PAG1	SOX9	SP100
ELF2	MYH3	RFC3	TGFB2	MMP8	PDCD6	SMAD1	PTEN
FGL2	MYC	RFWD3	MST1	PDIA4	SOD1	HPDL	TDP1

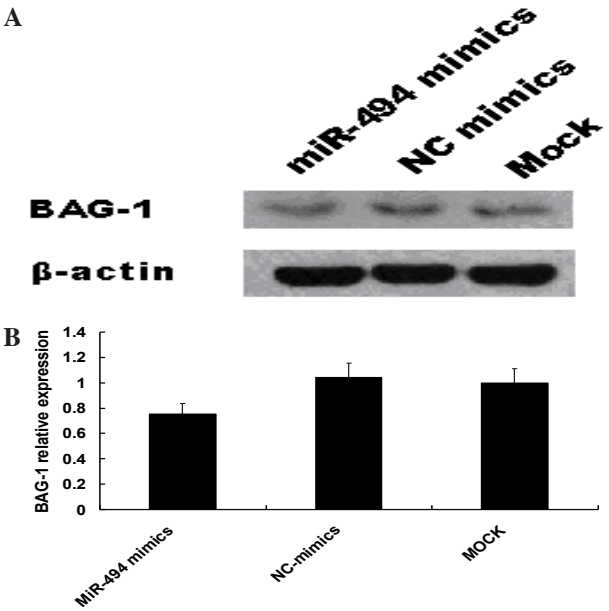

Figure 4. (A) The Expression of BAG-1 Protein in Cells Exposed to miR-494 Mimics; (B) Relative Expression of BAG-1 Protein in Cells Exposed to miR-494 Mimics

494 mimic. The results showed that compared with NM or M groups, the apoptosis rate was significantly increased in BGC-823 cells transfected with miR-494 mimic (Figure 3, *p<0.05).

Targeted genes of miR-494 prediction

We observed many target genes (Table 3), including BAG-1/BCL-10/PTEN/ PDCD-6, which suggested that miR-494 may be involved in the proliferation, apoptosis pathways.

Mir-494 can reverse the expression of BAG-1

To validate that miR-494 down-regulated the BAG-1 expression in BGC-823 cell, we used Western blot analysis to examine the expression of BAG-1 in BGC-823 cell transfected with miR-494 mimics. As is shown in Figure 6, the transfection of miR-494 mimics resulted in a significant decrease in BAG-1 protein level (Figure 4, *p<0.05).

miR-494 inhibits the expression of BAG-1 by targeting its 3'-UTR

To confirm the target relationship between miR-494 and BAG-1, we used bioinformatics tools TargetScan, PicTar, and Miranda to predict that BAG-1 was a target gene of miR-494 and identified the potential targeting sites of miR-494 at the position 1989 and 1982nt in the BAG-1 3'-UTR which was shown in Figure 5A. Next, to confirm that BAG-1 is the functional target gene of miR-494, We performed the firefly luciferase activity to examine the relative expression of BAG-1 luciferase activity in cells exposed to miR-494 mimics.The results

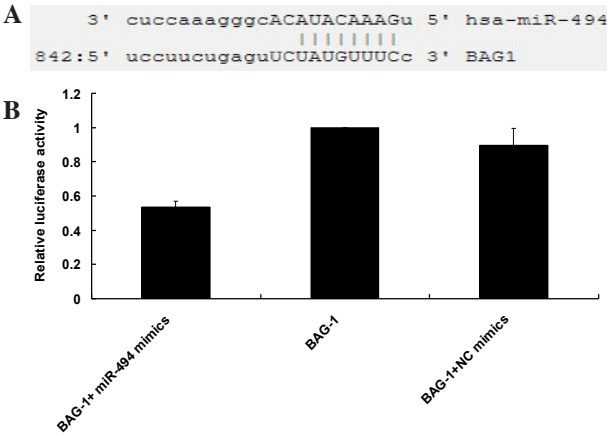

Figure 5. (A) The Predicted Binding Site Targeted by miR-494 in BAG-1 3'-UTR; (B) Relative Expression of BAG-1 Luciferase Activity in Cells Exposed to miR-494 Mimics

showed that the expression of BAG-1 luciferase activity in cells transfected with miR-494 was significantly decreased compared with BAG-1 group and BAG-1 in the cells transfected with NC mimics (Figure 5B, *p<0.05).

Discussion

Many evidence demonstrated that cinobufacin, a sterilised hot water extract of the dried toad skin, significantly inhibited the proliferation of many cancer cells (Zhai et al., 2013). In our previous studies, cinobufacin gradually decreased the cell proliferation rate of BGC-823 in a time- and dose-dependent manner with the increase of concentration and prolongation of action time, which was supported by the results of the previous studies (Han et al., 2008).

Accumulating evidence showed that cinobufacin was considered to have great effect on tumors by many mechanisms, such as induction of differentiation and apoptosis, inhibition of proliferation and cancer angiogenesis, reversal of multi-drug resistance, regulation of immune response, and disruption of cell cycle (Han et al., 2007). However, to date, the mechanism about the association of miRNA with cinobufacin in inhibiting the gastric cancer cells was poorly understood in previous study. In this study, depending on miRNA microarray, we showed that miRNAs were differently expressed in BGC-823 cells treated with cinobufacin, and miR-494 was significantly upregulated, which may play a major role in inhibiting the proliferation of BGC-823 cells. Therefore, we chose miR-494 as a candidate miRNA.

MiRNA-494, located on chromosome 14q32.31, has been shown to be up-regulated in retinoblastoma; However, functional importance of miR-494 in the context of cell proliferation and differentiation remains to be investigated. Mounting evidence showed that miR-494, considered to be a tumor-suppressing miRNA, suppressed the cell proliferation in many solid tumors including squamous cell carcinoma of the head and neck, B-Cell Lymphomas and so on (Chang et al., 2008; Robertus et al., 2010). However, no data to date are available about the association of miR-494 with gastric cancer . To identify

the contribution of miR-494 to the inhibition of gastric cancer cells, we transfected miR-494 mimic to BGC-823, the data demonstrated that miR-494 inhibited the BGC-823 cells proliferation and induced the cells apoptosis.

Many studies have demonstrated that MMP-9 (Hassan et al., 2012; Zhang et al., 2012; Akdeniz et al., 2013; Asuthkar et al., 2013; Darakhshan et al., 2013; Li et al., 2013), ERK1/2 (Romano et al., 2012), KIT (Kim et al., 2011) and PTEN (Liu et al., 2012) are considered to be the potential targeted genes of miR-494. In present study, BAG-1 is predicted as the targeted gene of miR-494 by using bioinformatics tools TargetScan, PicTar, and Miranda. The Bag-1 protein, identified because of the interaction with Bcl-2, and shown to enhance Bcl-2-mediated cell survival (Takayama et al, 1995) is deregulated in a variety of human tumors, including cancers of the breast, lung, colon, esophagus, larynx, oral cavity and tongue (reviewed by Wood et al, 2009), .In our studies, the results of western blot analysis and luciferase reporter assay validated that miR-494 inhibited the expression of BAG-1 by directly targeting 3' UTR of BAG-1. Therefore, it is reasonable to expect that miR-494 inhibits the BGC-823 cell growth and promotes cell apoptosis by targeting BAG-1, .

In conclusion, our findings highlight the molecular mechanism by which cinobufacin may inhibit the proliferation of BGC-823 by inducing the expression of miR-494, repressing BAG-1 expression which is the important component of the apoptosis signal transduction. MiR-494 may be a potential molecular target for cinobufacin in gastric cancer therapy.

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