AM22 Inhibites the Proliferation of Colorectal Cancer by Targeting CBFB

Fanyi Meng
Soochow University
Jiawei Li
Soochow University
Yajing Qiu
Peking Union Medical College Hospital
Haiyang Zhang
Soochow University
HJ Zhang (zhanghongjian@suda.edu.cn)
Soochow University
Weipeng Wang
Soochow University

Research Article

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Abstract

Our previous studies have revealed the important roles of the non-seed regions of miRNAs in gene regulation, which provided a novel insight in the development of miRNA analogs for cancer therapy. Here, we altered each nucleotide in the non-seed region of miR-34a and obtained novel synthetic miRNA analogs. Among them, AM22 with a base alteration from G to C at the 17th nucleotide of miR-34a, showed extensive anti-proliferative activity against several colorectal tumor cell lines, and achieved effective inhibition of CBFB (core binding factor subunit β) expression. Subsequent investigations demonstrated that AM22 directly targeted CBFB by binding to its 3'-untranslated region (3'-UTR). Inhibition of CBFB showed obvious anti-proliferative activity on HCT-116 and SW620 cells. Furthermore, the anti-proliferative effects of AM22 on these cells were also measured in the xenograft mouse models. In conclusion, this study identified AM22 as a potential anti-tumor miRNA by targeting CBFB, and provided a new design approach for miRNA-based cancer treatment by changing the non-seed region of miRNA.

Introduction

Colorectal cancer (CRC) has become the third most common cancer in the world, and it is showing a trend of younger age (1). The prognosis of CRC is better with an earlier diagnosis. The outcome of CRC may also be improved by targeting pathways involved in colorectal cancer progression, such as anti-epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) based therapeutics (2). Therefore, there is great clinical value to find early biomarkers and new therapeutic targets for CRC.

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules, playing essential roles in a wide range of physiological processes(3). In cancer, the majority of studies on miRNAs have focused on their function as an oncogene or tumour suppressor(4). Dysregulation of miRNA expression has been reported in most cancer types(5, 6),and miR-34a is one of the dysregulated miRNAs that has attracted much attention. Up to now, there are more than 800 targets of miR-34a have been verified by experiments, and most of these genes are related to the promotion of cancer cell proliferation, apoptosis, migration and immune evasion(7, 8). miR-34a is a pivotal miRNA whose expression is transcriptionally controlled by the tumor suppressor p53 in physiological conditions(9), and is closely related to the occurrence and development of CRC(10–13). A number of studies had confirmed that restored the abnormally low expression of miR-34a in CRC has a good anti-tumor effect *in vivo* and *in vitro*(13, 14). Previously we have provided a novel synthetic miRNA treatment for CRC by modifying the sequence of miR-34a(15).

miRNAs exert their regulatory effects by binding to the 3’-untranslated region (3’-UTR) of target genes, with an optimal complementarity of 2–8 nucleotides (the seed region) in the 5-prime of miRNAs(16). However, the sequences outside the seed region may also be capable of impacting miRNA-mediated gene regulation(17). Usually, the miRNAs in the same family share the seed region but differ in the non-seed regions, particularly the 3’-distal end, which results in different gene regulation patterns. While it is well

documented that a single nucleotide alteration (or single nucleotide polymorphism) in the seed region has a profound effect on target gene regulation(18, 19), the importance of structural alterations of non-seed regions has only been recently recognized(20, 21). It is thus hypothesized that a given miRNA-mediated regulation could be modified by single nucleotide alteration in the non-seed regions.

In this study, we used miR-34a as a template and performed a G > C substitution at the 17th base of the distal region, thus obtaining a new miRNA named AM22, with excellent anti-tumor activities both *in vivo* and *in vitro*. Mechanistic studies demonstrated that AM22 bond to CBFB 3'-UTR and inhibited its protein translation. In addition, inhibition of CBFB had obvious anti-proliferative activity on HCT-116 and SW620 cells as well as their xenograft models. Our research provided a theoretical basis for the development of new miRNA-based cancer treatment.

Materials And Methods

Cell culture and reagents. Cell lines such as SW 620, HCT-8, HCT-116, SW 480, A549, H460, Panc-1, SGC-7901 and CHO were purchased from the American Type Culture Collection (Manassas, VA) and cultured under the conditions recommended by the vendor. CBFB siRNA, the mimics, mimics control, agomir and agomir control were synthesized by GenePharma (Suzhou, China). CBFB/GV141 vectors and vector control were synthesized by GeneWiz (Suzhou, China). DMEM and RPMI 1640 cell culture media were purchased from Gibco (Thermo, Massachusetts, USA).

Cell Transfection. To investigate the regulatory role of mimics in gene expression, synthetic DNAs or RNAs (mimics or mimics control; Table S1) were transfected into CRC cells using lipofectamine 2000 (Invitrogen, California, USA) for 48 h (RNA analysis) or 72 h (protein analysis).

RNA sequencing (RNA-seq). This assay was performed by Shanghai Oebiotech Co., Ltd. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion, Texas, USA). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent, California, USA). The libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, California, USA). Then the libraries were sequenced on HiSeq™ 2500 sequencing platform (Illumina, California, USA) and 125bp/150bp paired-end reads were generated.

RNA quantification. For quantitative real-time PCR (qPCR), total RNA was isolated from cells using TRIzol reagent (Takara, Tokyo, Japan). The total RNA extracted was reverse transcribed into cDNA using NxGen M-MuLV reverse transcriptase (Invitrogen, California, USA). qPCR was performed using quantitative RT-PCR master mix (Bio-Rad, California, USA) and primers (Table S1) on CFX96 Touch™ real-time PCR system (Bio-Rad, California, USA). RNA expression levels were normalized to those of GAPDH.

Western blot. Total proteins from cells and tumor tissues were extracted using RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo, Massachusetts, USA). Alliquots of 20 μg proteins were separated on a 10% SDS-PAGE gel and electro-transferred onto a PVDF membrane. After blocking using 5% skim milk, the membrane was

incubated with primary antibody at 4°C overnight and subsequently with the corresponding secondary antibody (Santa Cruz Biotech, Texas, USA) for 1 h at room temperature. The membrane was then developed using Clarity Western ECL substrates (Merck Millipore, Darmstadt, Germany) and visualized with a ChemiDocTM MP Imaging System (Bio-Rad, California, USA). Protein expression was normalized with GAPDH expression. The antibodies against CBFB (#62184S) and β-Actin (#3700S) were purchased from Cell Signaling Technology (Massachusetts, USA).

Dual-luciferase reporter (DLR) assay. 3'-UTR of CBFB gene was cloned downstream of the pGL3-control vector (Promega, Utah, USA)) using *Xba*I and *Hpa*I endonucleases (NEB, Massachusetts, USA). CHO cells were co-transfected with 200 ng pGL3 constructs and 100 nM mimics (GenePharma, Suzhou, China) using lipofectamine 2000 (Invitrogen, California, USA). Luciferase activity was measured after 24 h using the DLR assay system (Promega, Utah, USA).

CCK-8 assay. Cell proliferation was measured using the CCK-8 assay kit (Dojindo, Kyushu, Japan). Approximately 3000 cells were plated into each well of a 96-well plate (Corning, New York, USA) and transfected with 50 nM miRNA mimics using lipofectamine 2000. After 72 hours, 10 μL CCK-8 was added into 90 μL culture medium. Cells were subsequently incubated for 15 min at 37°C and the optical density was measured at 450 nm using M3 SpectraMax microplate reader (Biotek,Vermont, USA).

Tumor xenograft model. Protocols for animal husbandry and experiments were approved by the Institutional Animal Care and Use Committee at Soochow University. All animal experiments were complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Male Nude mice were purchased from SLAC int. (Shanghai, China). All animals were kept in specific-pathogen-free (SPF) conditions in the Animal Resource Center at Soochow University.

To investigate anti-tumor activities of miR-34a and AM22, HCT-116 cells (3×10^6) or SW620 cells (5×10^6) were subcutaneously inoculated in the lower right flank of mice. Tumor-bearing mice were randomly assigned to different groups after tumors reached a volume of 100–150 mm^3. An intravenous dose (1.45 mg/kg) of miR-34a agomir, AM22 agomir or agomir NC was administered once every 2 days. The tumor volume was detected by using vernier caliper every 3 days after inoculation. Tumor tissues were collected at the end of the study for analysis of CBFB protein.

Data analysis. Statistical analyses were conducted using Student's t-test and Pearson Correlation Coefficient. The results were presented as mean ± SD, with $P < 0.05$ considered as statistically significant.

Results

AM22 with a nucleotide substitutions in the non-seed region of miR-34a has more potent anti-tumor activity than miR-34a

miR-449 and miR-34 family members share the same seed sequence. To explore the effect of nucleotides in the non-seed region of miR-34a on gene regulation, we selected miR-34a as a template, and the sequence following the common seed 5'-GGCAGUGU-3' of miR-449 and miR-34 families were replaced in turn by single nucleotide. Thirty-nine miR-34a analogs are generated and named with AMn (Fig. 1A, Table S1). Subsequently, we tested the anti-tumor activities of the analogs in HCT-116 cells. As shown in Fig. 1B, the analog numbered AM22 had the strongest anti-tumor effect. Moreover, AM22 also exhibited more potent anti-tumor activities than miR-34a in the colorectal cancer cell lines HCT-8, SW480, SW620 and LoVo, except for Caco-2 (Fig. 1C), as well as in non-small cell lung cancer A549 and H460, pancreatic cancer Panc-1, and gastric cancer SGC-7901 (Fig. S1).

CBFB is a direct target gene of AM22

To investigate the gene regulation mediated by AM22 or miR-34a, we transfected AM22 and miR-34a into HCT-116 cells, respectively, and determined the gene expression by RNA-seq analysis. As shown in Fig. 2A, the number of AM22-regulated genes was quite different from that of miR-34a. As for the down-regulated genes (change fold ≥ 2; $P < 0.05$), only 306 of 1096 genes were significantly down-regulated by either AM22 or miR-34a. In the 790 genes down-regulated by AM22, 8 genes were predicted to be the target genes of AM22 by TargetScan (www.targetscan.org), including PGK1, SMAD5, and CBFB etc (Fig. 2A). The effects of AM22 on the expression of the eight genes were detected by quantitative real-time PCR assays. The results showed that SMAD5, CBFB, RNF128 and PIK3CA were down-regulated by AM22, which is in line with the results of RNA-sEq. Among them, We finally chose CBFB for the next research. The binding partens between CBFB 3’-UTR and AM22 or miR-34a were predicted by TargetScan. The result showed that AM22 had an additional complementary base-pair at 17th nucleotide as compared with miR-34a (Fig. 2B). Then we tested the effects of AM22 and miR-34a on the expression of CBFB mRNA and found that only AM22 reduced the level of CBFB mRNA in concentration-dependent manner (Fig. 2C). Meanwhile, we found that the protein levels of CBFB were also significantly dereased by AM22 instead of miR-34a (Fig. 2E). Furthermore, the results of the dual-luciferase report assays showed that only AM22 could directly bind to CBFB 3'-UTR (Fig. 2D). Interestingly, miR-34a enhanced the activity of luciferase (Fig. 2D), which is consistent to the effect of miR-34a on the mRNA level of CBFB at 100 nM. However, the mechanisms are required to be further investigated. Nevertheless, these findings provided evidences to demonstrate that CBFB is the target gene of AM22 rather than miR-34a.

CBFB inhibition impacts CRC cell proliferation in vitro.

To explore the roles of CBFB in the occurrence and development of CRC, we first analyzed the data in the TCGA database and found that the expression of CBFB in colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), pancreatic adenocarcinoma (PAAD), stomach adenocarcinoma (STAD) and cholangiocarcinoma (CHOL) were significantly higher than those in the normal tissues (Fig. 3A). Moreover, the highly expressed CBFB was also observed in each cancer stage of colorectal cancer (Fig. 3B). Next, we synthesized two CBFB siRNAs and two overexpression plasmids to further explore its function in CRC (Fig. 3C). As shown in Fig. 3D, inhibition of CBFB had significant anti-tumor activities in

both HCT-116 and SW620 cells in a time-dependent manner. While CBFB overexpression significantly enhanced the proliferation of these two cell lines in a time-dependent manner (Fig. 3E). These findings demonstrate that CBFB promotes the proliferation of CRC cells.

AM22 Inhibits the growth of HCT-116 and SW620 xenografts

We then tested the inhibitory roles of AM22 in tumor growth *in vivo*. We subcutaneously grafted HCT-116 and SW620 cells into the lower back of nude mice. After palpable tumors had formed, the tumor-bearing mice were intravenously injected with 1.45 mg/kg/day of AM22 or miR-34a agomir for once every 2 days. As shown in Fig. 4A and 4B, AM22 significantly inhibited the growth of HCT-116 and SW620 tumors, and it has a more potent efficacy on HCT-116 tumorscompared with miR-34a agomir. Western blots of the tumorsshowed that CBFB was markedly inhibited by AM22 instead of miR-34a (Fig. 4C and 4D).

Discussion

As we know, the seed region of miRNA (usually 2–8 nucleotides) binds to the 3'-UTR of target mRNA to regulate gene expression(16). Several studies have confirmed that nucleotide changes in the seed region led to alteration in gene regulation partens(21, 22). However, the roles of the non-seed regions of miRNAs in the gene regulation is still unclear. In fact, studies had confirmed that increased number of complementary base pairs in non-seed regions of miRNAs could compensate for base mutations in seed regions(20, 23). In addition, changes in the number and form of complementary base pairs in non-seed regions can generate new miRNAs for targeting specific genes(18, 19). These results indicated that, in addition to the seed region, the non-seed region could also affect the regulatory activities of the miRNA.

CBFB is a cofactor of the Runx transcription factor family (Runx1, Runx2, and Runx3), which mainly regulates hematopoiesis (eg. RUNX1) and osteogenic (eg. RUNX2) specific genes(24). CBFB has recently emerged as a highly mutated driver in a variety of human cancers including breast cancer, although the significance of these mutations has not yet been determined(25, 26). In addition, CBFB and RUNX1 mutations have been identified as drivers of many types of cancers, including breast(25), ovarian(27) and prostate cancer(28). However, the roles of CBFB in the other cancers are still unknown, for instance, colorectal cancer.

In the present study, several new synthetic miRNA analogs with the same seed region as miR-34a displayed different anti-proliferation activities on HCT-116 cells. By altering a nucleotide in the non-seed region of miR-34a, a newly generated miRNA named AM22 directly bond to CBFB 3’-UTR and inhibited its expression at the mRNA and protein levels. Additionally, CBFB has been proved to be a triggering gene in the progression of CRC. Subsequent investigations demonstrated that AM22 had potent antitumor activities *in vitro* and *in vivo* superior to miR-34a. The stronger antitumor activity of AM22 than miR-34a was proved to be derived from inhibition of CBFB in HCT-116 and SW620 xenografts. These findings

further support the conclusion of a previous report describing the possibility that artificial miRNA-like molecules could be designed to target specific genes (29).

miR-34a was used as the template for the generation of miRNA analogs because that miR-34a is the first miRNA into clinical trials and the functions of miR-34a in cancer development has been investigated in a large number of preclinical studies(7, 11, 30). Since the failure of the phase I clinical trial was due to immune-related adverse reactions(31), the ongoing researches on miRNAs are focusing on their roles in the immune response(32, 33). In this study, we found that both the target genes and anti-tumor activity could be altered by nucleotide changes in the non-seed region of miR-34a. Thus we supposed that the replacement of nucleotides in the non-seed region of miR-34a might be a possible wayto improve the safety of miR-34a.

In conclusion, the current study revealed that by alternating nucleotide(s) in the non-seed region of miR-34a, novel synthetic miRNAs were generated. Among these miRNAs, AM22 demonstrated superior antitumor activities by suppressing CBFB expression. Therefore, our study provides a novel miRNA with potent anti-tumor activity and offers a strategy for generating new miRNAs for cancer therapy by altering nucleotide(s) in the non-seed region of miRNAs.

Declarations

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Conflict of interest: All authors declare no conflict of interest.

Availability of data and material: Sets of data or summaries generated during the present study are available from the corresponding author upon reasonable request.

Authors' contributions:

Conceptulization: Meng and Wang;

Study design and execution: Meng, Li and Qiu;

Data analysis and summary: Meng and Li;

Writing - original draft: Meng and Zhang;

Writing - review & editing: Meng, Zhang and Wang.

Ethics approval: Protocols for animal husbandry and experiments were approved by the Institutional Animal Care and Use Committee at Soochow University. All animal experiments were complied with the

ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Consent for publication: All authors are concent to publish this study in the journal of *Investigational New Drugs*.

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Figures

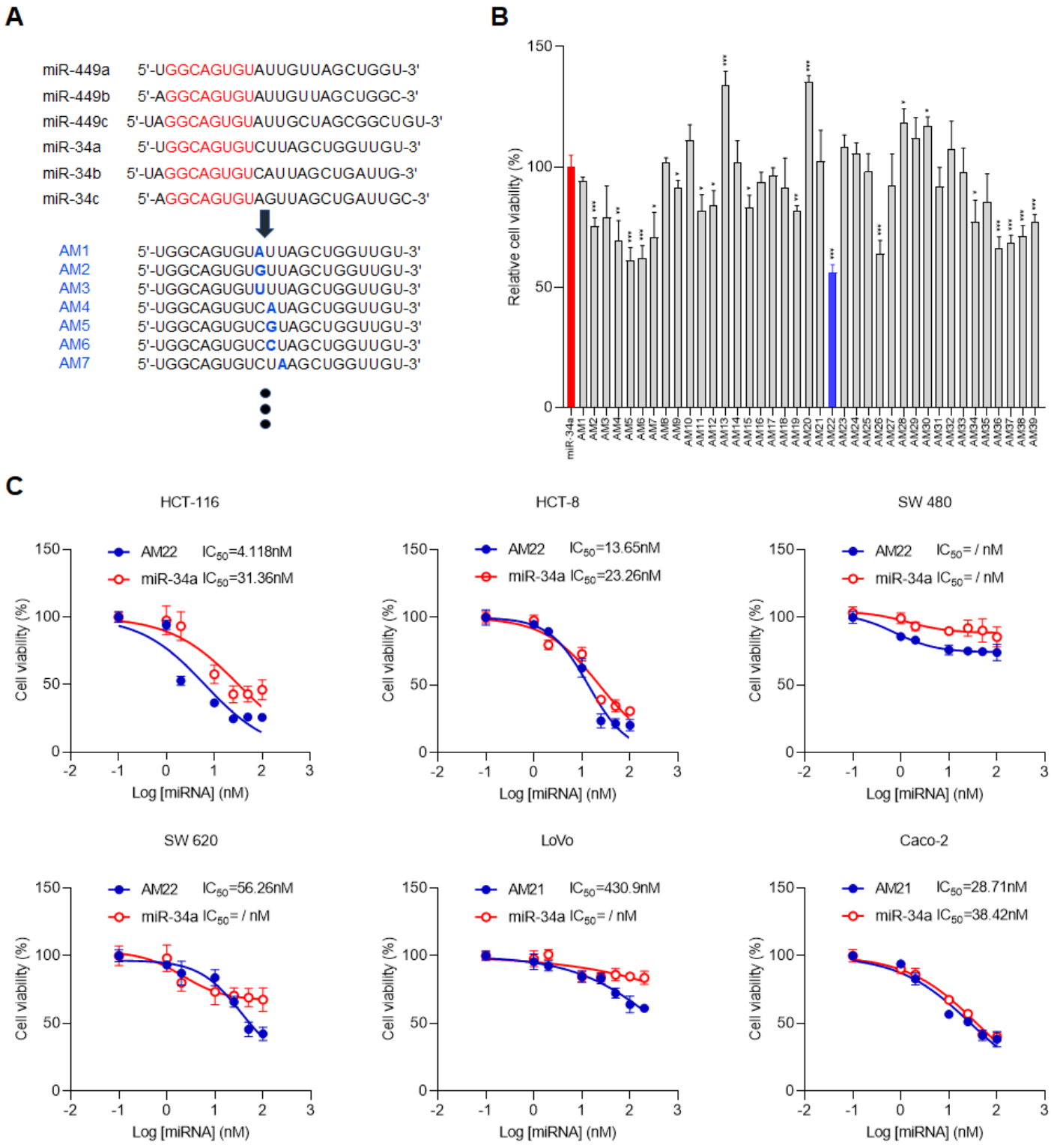

Figure 1

The effects of nucleotide changes in the non-seed region of miR-34a on the anti-tumor activities. (A) The sequences of miRNAs in the miR-34 / miR-449 families and the miR-34a analogs. (B) The inhibitory roles of miR-34a (50nM) and its analogs (50nM) in the proliferation of HCT-116 cells. (C) The inhibitory roles of miR-34a and AM22 in the proliferation of HCT-116, HCT-8, LoVo, Caco-2, SW480, and SW620 cells. Data

represent mean ± SD. Significance compared to negative control was assessed by two-sided t-test. ***P<0.001; **P<0.01; *P<0.05; ns, no significance.

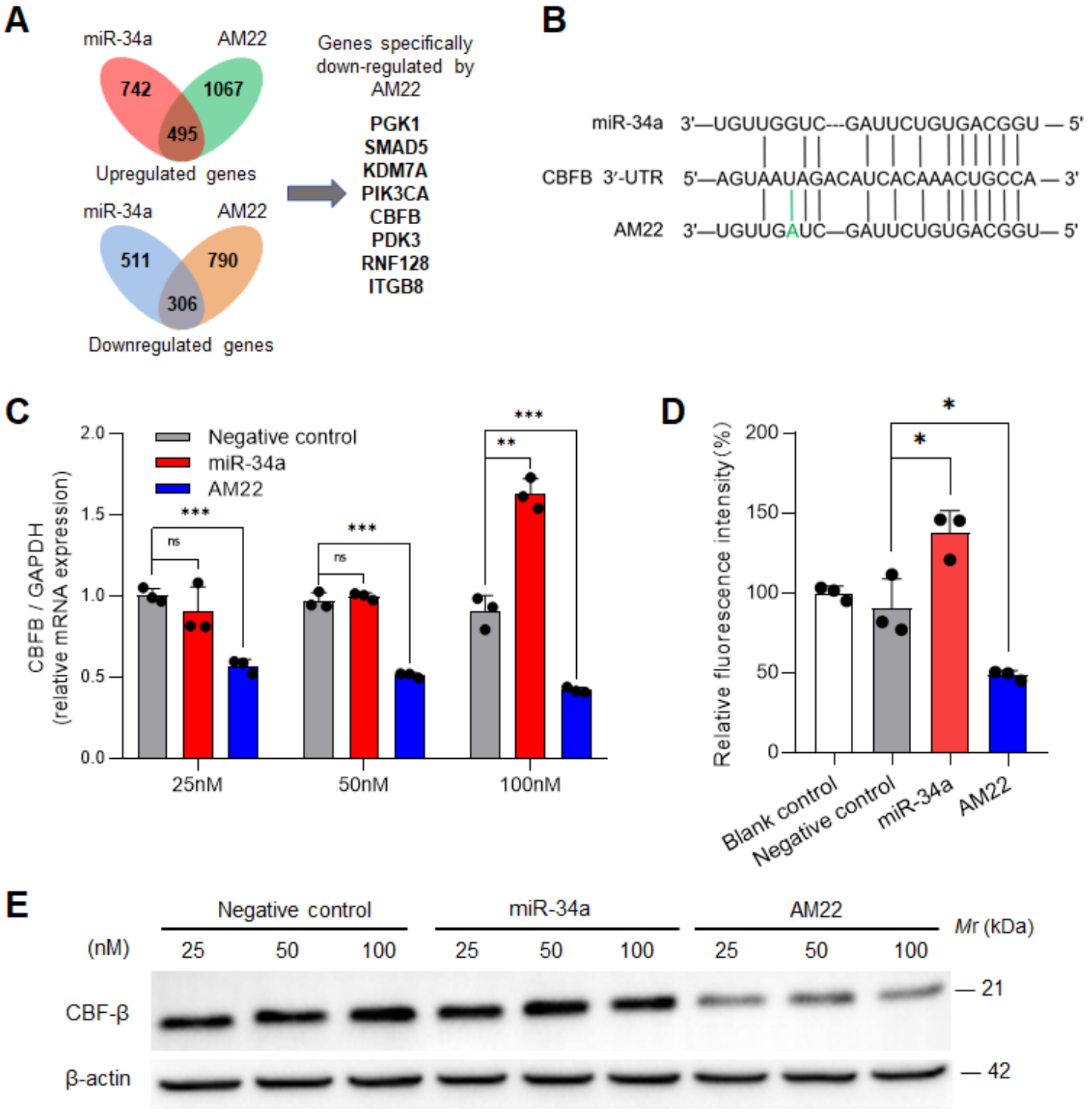

Figure 2

The regulatory role of AM22 in CBFB expression. (A) Venn-diagram displayed the overlap in the number of genes dysregulated by AM22 or miR-34a, and 8 candidate genes were identified. (B) The predicted binding-sites of miR-34a and AM22 in CBFB 3'-UTR. (C) Real-time PCR data showed the mRNA expression of CBFB in HCT-116 cells transfected with different concentrations of AM22 or miR-34a . (D) Luciferase

reporter assays showed that AM22 directly bond to CBFB 3'-UTR. (E) Western blot data showed the expression of CBFB in HCT-116 cells transfected with different concentrations of AM22 or miR-34a. Data represent mean ± SD. Significance compared to negative control was assessed by two-sided t-test. ***P<0.001; **P<0.01; *P<0.05; ns, no significance.

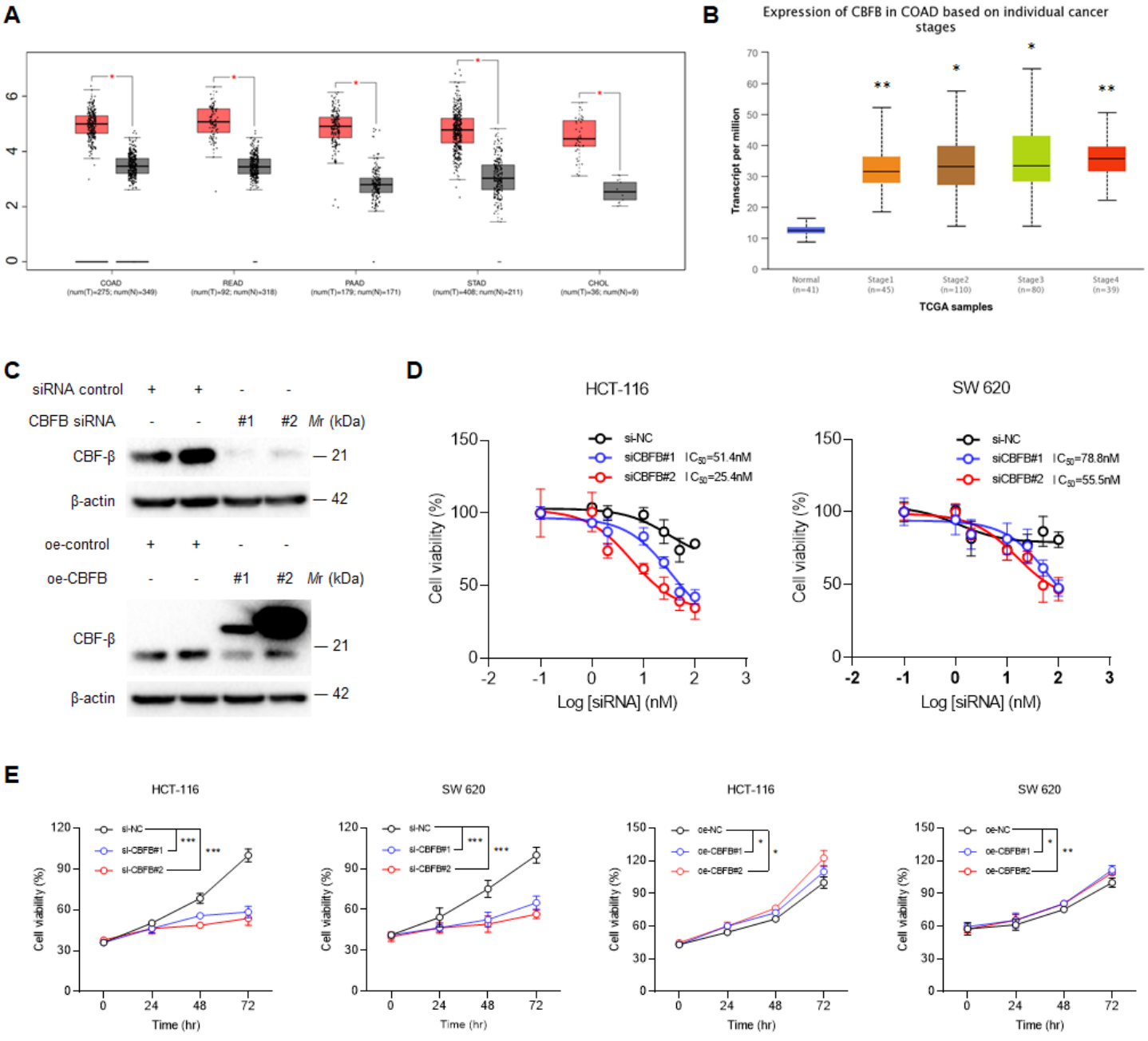

Figure 3

Impact of CBFB inhibition on CRC cells proliferation in vitro. (A) Expression of CBFB in cancer tissues from TCGA samples. (B) TCGA data showed the expression of CBFB in COAD at individual cancer stages. (C) Western blot data showed the expression of CBFB in HCT-116 cells after treatmented by siRNA (50nM) or over-expression plasmid (200ng). (D) The inhibitory roles of CBFB siRNA in the proliferation of HCT-116 and SW620 cells. (E) The inhibitory roles of CBFB siRNA and over-expression plasmid in the

proliferation of HCT-116 cells. Data represent mean ± SD. Significance compared to negative control was assessed by two-sided t-test. ***P<0.001; **P<0.01; *P<0.05; ns, no significance.

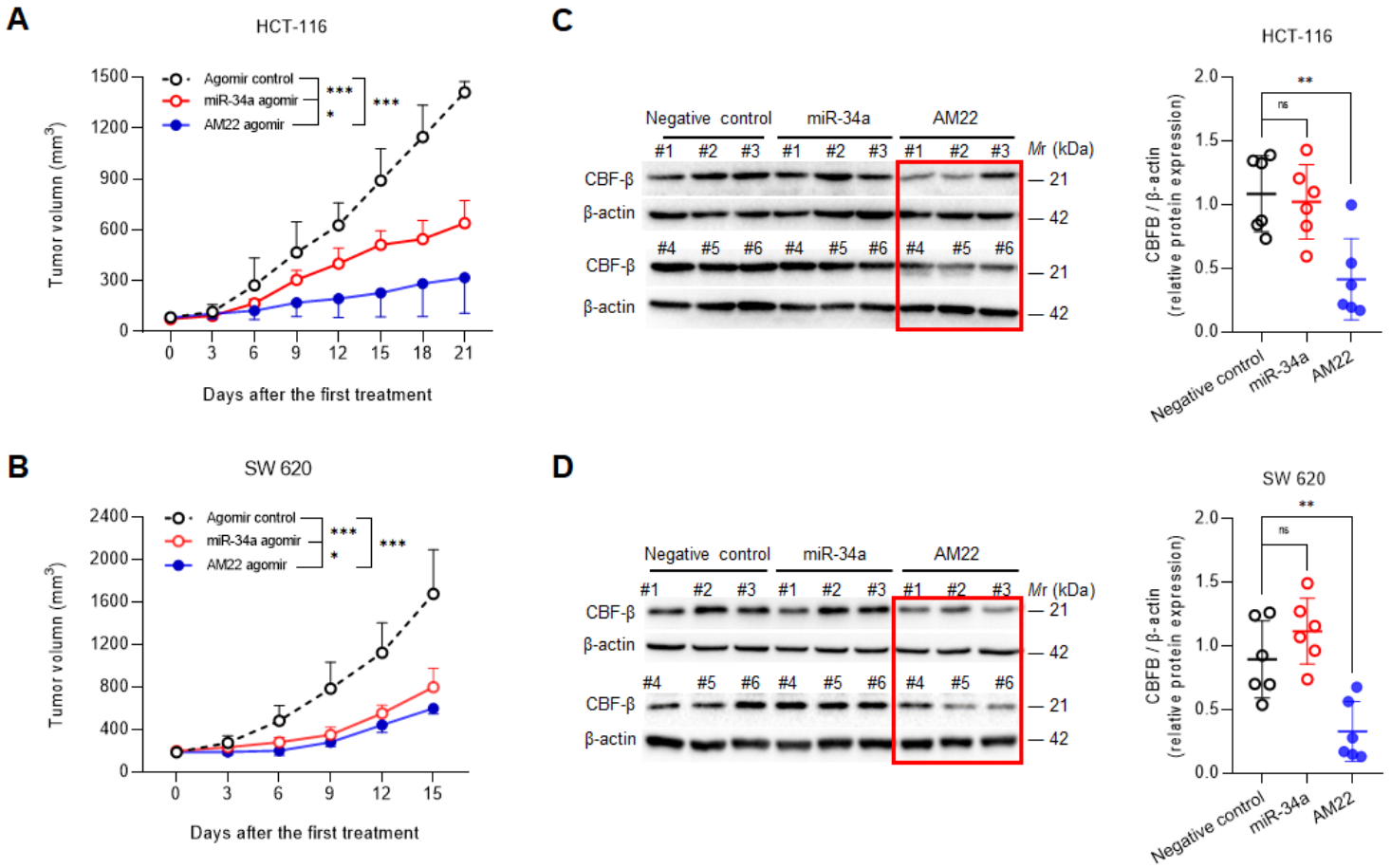

Figure 4

AM22-mediated inhibition of tumor growth in HCT-116 and SW620 xenografts. (A) The inhibitory roles of miR-34a agomir and AM22 agomir in the growth of tumors in nude mice (n=6). The doses of agomir were 1.45 mg/kg for once every 2 days. (B) The expression of CBF-β in the xenografts after treatments with miR-34a agomir or AM22 agomir. Data represent mean ± SD. Significance was assessed by two-sided t-test. ***P<0.001; **P<0.01; *P<0.05.

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