Downregulation of microRNA-1 and microRNA-145 contributes synergistically to the development of colon cancer

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Abstract. The aim of the present study was to identify the differentially expressed microRNAs (miRNAs or miRs) in patients with colon cancer and examine their roles in the pathogenesis of the disease. The expression profiles of miRNAs were examined in tumor tissue samples collected from 20 patients with histologically confirmed colon cancer and in the adjacent non-cancerous control tissues using microarray analysis. We found that numerous miRNAs were differentially expressed in the colon cancer tissues compared with the control tissues. Following functional analysis, we noted that three miRNAs which were significantly downregulated, miR-145, miR-451 and miR-1, shared the same target gene, [NLR family, apoptosis inhibitory protein (NAIP)], which has previously been reported to be involved in the development of colon cancer. We then confirmed that NAIP is a target gene of miR-145 and miR-1, but not of miR-451, using a luceriferase assay, and we confirmed the expression patterns of these miRNAs and NAIP in the tumor tissue, as well as in the adjacent non-cancerous control tissues. Additionally, in order to examine the function of miR-145 and miR-1 in human colon cancer, we transiently transfected a human colon cancer cell line with miR-145 and miR-1 mimics or inhibitors, and the results of MTT assay revealed that the re-expression of miR-145 and miR-1 inhibited the survival of colon cancer cells, which may be attributed to the inhibition of the anti-apoptotic activity of NAIP. Our findings demonstrated that miR-145 and miR-1 play a negative regulatory role in the proliferation of colon cancer by targeting NAIP; thus, miR-145 and miR-1 may prove to be novel therapeutic targets in the treatment of colon cancer.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide (1). Even though the survival rate has doubled over the past 20 years, it remains one of the most fatal malignancies worldwide (2-4). CRC is a heterogeneous disease, and a complex series of molecular changes have been reported to be involved in the transformation of the normal colonic mucosa into a potentially invasive tumor over a long period of time (5). The development of CRC is generally believed to result from complex interactions between genetic and environmental factors (6). Molecular biomarkers based on gene mutations located in oncogenes or tumor suppressors, such as adenomatous polyposis coli (APC), v-raf murine sarcoma viral oncogene homolog B1 (BRAF) and p53 have been established for over a decade (6); however, the clinical applications of these biomarkers remain limited. For this reason, the development of novel molecular markers is necessary in order to improve the diagnosis, prognosis and treatment of CRC.

MicroRNAs (miRNAs or miRs) are a class of small non-coding RNAs, approximately 22 nucleotides in length, that post-transcriptionally regulate the expression of up to 1/3 human genes. miRNAs target mRNAs through imperfect base pairing of the 5'-end of miRNAs to multiple sites in the 3'-untranslated regions (UTRs) of the target transcript, and this imperfect miR-mRNA hybrid with central bulges (9-12 nt) recruits a microRNA ribonucleoprotein complex (miRNP) that enables the translational inhibition or exonucleolytic mRNA decay (7). More than half of the annotated human miRNAs have been found to be located in tumor-related chromosome segments or fragile hot spots that are predisposed to translocation, insertion or deletion in a number of human malignancies, including colon cancer (8), and thus, some miRNAs can function either as oncogenes or tumor suppressors (9-11).

Using microarray technology, we can detect the expression levels of thousands of genes simultaneously, which makes it a promising tool with which to discover novel potential diagnostic or therapeutic biomarkers, and it can assist us in exploring the underlying molecular mechanisms (12,13). A growing body of evidence indicates that DNA microarray profiling performed on clinical specimens may be directly applied to cancer diagnosis, as well as to the evaluation of prognosis (14,15). Additionally,

expression profiling has been used to predict the response to cancer treatment (16). Expression profiling analysis has also been used to establish characteristic miRNA signatures that can predict the clinical outcomes of various malignancies (17).

In order to broaden our understanding of the molecular mechanisms underlying the pathogenesis of human CRC and explore novel potential biomarkers for use in clinical practice, in this study, we examined and compared gene expression profiles in CRC tissues and adjacent non-cancerous control tissues obtained from surgical resections, and using functional analysis, we focused on the differentially expressed miRNAs, their potential target gene, and their roles in regulating colon cancer cell behavior.

Materials and methods

Tissue sample collection. Paired primary CRC samples and adjacent histologically normal tissues were collected from a total of 60 patients with CRC in the Department of General Surgery at The Third Affiliated Hospital of Guangzhou Medical University (Guangdong, China). Tumor tissues and adjacent non-cancerous tissues that were at least 2.0 cm distal to the tumor margins were snap-frozen in liquid nitrogen and then stored at -80°C until use. All samples were evaluated by two pathologists, according to the World Health Organization (WHO) guidelines (18). Those who had received chemotherapy or radiotherapy prior to surgery were excluded from this study. Informed consent was obtained from all patients, and the study was approved by the Human Research Ethics Committee of Guangzhou Medical University.

Gene expression analysis and data processing. RNA was isolated from each tissue sample and subjected to gene expression analyses using whole genome expression microarrays (Funeng Biotech Co., Ltd., Guangzhou, China). Following array data processing, differentially expressed genes were identified using the GeneSpring GX software package (Funeng Biotech), and only those miRNAs with differences >1.6-fold between the 2 groups (normal and tumor tissues) are listed in Table I.

In silico analysis. The target genes of the miRNA or the regulatory miRNA of certain genes were identified by searching online miRNA databases (www.TargetScan.org and www.miRanda.org). The candidate gene list was shortened based on their pathophysiological function.

RNA isolation and quantitative PCR (qPCR). Total RNA was isolated from HCT116 cells and tissues using the total RNA isolation kit (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA using the SuperScript III kit (Invitrogen). Total RNA concentrations were measured using a NanoDrop ND-1000 fluorospectrometer (NanoDrop Technologies, Wilmington, DE, USA). To confirm the differential expression of the selected miRNAs identified in our microarray analyses, standard SYBR-Green-based qPCR (Sigma-Aldrich, St. Louis, MO, USA) analyses were conducted. Specific primer sequences were as follows: miR-1 forward, 5'-aggcaaagagaatagttccccag-3' and reverse, 5'-cggacttcgt ggagatgga-3'; miR-145, forward, 5'-ctggctcttgataccccct-3' and reverse, 5'-tcaacactgagacgggctcc-3'; miR-451, forward, 5'-ggaaa

ccaggaagcctagca-3' and reverse, 5'-tgattcagtgccattt tgcc-3'. Fluorescence was measured at the final step of each cycle using a real-time PCR system (ABI-7900; Applied Biosystems, Foster City, CA, USA). The housekeeping gene, U6, was used to normalize the expression data for the gene of interest.

Luciferase reporter assay. The human NLR family, apoptosis inhibitory protein (NAIP) 3'-UTRs were amplified by PCR and cloned into a modified version of pcDNA3.1(+) that contained a firefly luciferase reporter gene (Invitrogen), at a position downstream of the luciferase reporter. The vectors were termed wild-type 3'UTRs, and the primers for cloning the 3'-UTRs of NAIP were as follows: sense, 5'-GAT GAA TTC TTA TCC CCT GCC CCT TCC-3' and antisense, 5'-TAT CTC GAG TGG GTC CAC CAT GGC TAA GTG A-3'. Site-directed mutagenesis of the miRNA binding sites in the NAIP 3'UTR was performed using a Site-Directed Mutagenesis kit (SBS Genetech, Beijing, China) and termed mutant-1, -2 and -3 3'UTRs, as shown in Fig. 2. All constructs were confirmed by DNA sequencing. CRC cells (HCT116) were grown in a 48-well plate and co-transfected with 400 ng of either individual miR mimics (miR-1, miR-145 or miR-451) or the control, 40 ng of the firefly luciferase reporter plasmid including the 3'-UTR of the target gene, and 4 ng of pRL-TK, a plasmid expressing *Renilla* luciferase (Promega, Madison, WI, USA). After 24 h, the cells were collected and the luciferase activity was determined using a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA).

Cell cycle and apoptosis analysis. At 48 h following transfection, the cells were collected by trypsinization and washed with phosphate-buffered saline (PBS). An Annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany) was used to stain the cells, according to the manufacturer's instructions. Apoptosis was evaluated through fluorescence-activated cell sorting (FACS) using CellQuest software (Becton-Dickinson and Company, Franklin Lakes, NJ, USA), and Annexin-V-FLUOS-positive cells were regarded as apoptotic cells. Flow cytometric analysis was performed using a Becton-Dickinson flowcytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis. The cells were lysed in RIPA lysis buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate] (from Beyotime, Nanjing, China). The lysates were then centrifuged at 12,000 x g/min at 4°C for 15 min, and were subjected to 10% SDS polyacrylamide gel electrophoresis. The separated proteins were then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and the membranes were subsequently incubated with primary antibodies at 4°C overnight and washed 3 times with PBS for 5 min, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:15,000 dilution; Cat. no. ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 1.5 h and detected with an ECL kit (Applygen, Beijing, China). The primary anti-NAIP antibody (Cat. no. sc-11064; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin antibody (Cat. no. sc-47778; Santa Cruz Biotechnology) were diluted at 1:2,000.

Table I. Differentially expressed microRNAs between cancerous tissues and adjacent non-cancerous control tissues, as identified by microarray analysis.

| Gene | Mean | | t | P-value |
	Normal tissues	Tumor tissues		
Downregulated				
hsa-let-7c	0.98	-0.43	3.809	0.001
microRNA-126	2.8	1.56	3.852	0.001
microRNA-451	1.91	0.27	4.153	0.001
microRNA-23b	2.7	1.14	4.394	<0.001
microRNA-1979	1.79	0.47	4.453	<0.001
microRNA-27b	1.3	-0.19	4.543	<0.001
hsa-let-7b	1.1	-0.52	5.702	<0.001
microRNA-125b	1.43	-0.37	6.202	<0.001
microRNA-768-3p	3.34	1.56	6.564	<0.001
microRNA-99a	3.79	1.83	6.73	<0.001
microRNA-4770	3.17	0.91	7.162	<0.001
microRNA-125a-5p	2.66	0.84	7.195	<0.001
microRNA-1	6.47	2.68	7.218	<0.001
microRNA-100	3.45	1.58	7.294	<0.001
microRNA-143	2.3	-0.5	7.389	<0.001
microRNA-145*	8.42	4.99	7.897	<0.001
Upregulated				
hsa-let-21	0.96	-0.49	3.901	0.001
microRNA-128	2.5	1.48	3.912	0.001
microRNA-375	2.01	0.24	4.024	0.001
microRNA-26b	2.81	1.09	4.283	<0.001
microRNA-146a	1.81	0.46	4.503	<0.001

Cell culture. The human CRC cell line, HCT116, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 lg/ml streptomycin (Invitrogen), at 37°C in a 5% CO_2 atmosphere.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HCT116 cells were transfected with miR-1 mimics and/or miR-145 mimics, or miR-1 inhibitors, or negative control (NC) and incubated for 48 h. MTT soloution (25 μl 0.5%; Sigma-Aldrich) was added to each well followed by incubation for a further 3 h. The medium was then replaced with 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich), which was added to the microplate, and shaken on a rotary platform for 15 min. The cell survival rate was determined by measuring the optical density (OD) values at a 492 nm wavelength and comparing these values with the OD values of the controls.

RNA oligoribonucleotide and transfection. MicroRNA mimics were purchased from Dharmacon (Lafayette, CO, USA): miR-145

mimic (MIMAT0000437), miR-1 mimic (MIMAT0000416) and miR-451 mimic (MIMAT0001631). miRNA inhibitors were purchased from Guangzhou RiboBio Co., Ltd., Guangzhou, China: miR-1 inhibitor (miR10005824-1-2) and miR-145 inhibitor (miR20000437-1-5). The HCT116 cells were cultured to 70% confluence, and Lipofectamine 2000 (Invitrogen) was used to transfect the DNA or RNA.

Statistical analysis. Data are expressed as the means ± SD. Statistical analysis was performed using the SPSS 19.0 software package (IBM Inc., Chicago, IL, USA). P-values were calculated using the two-tailed Student's t-test or one-way ANOVA. P-values <0.05 were considered to indicate statistically significant differences, and P-values <0.01 were considered to indicate highly significant differences.

Results

Microarray analysis of miRNAs differentially expressed in cancer and adjacent non-cancerous tissues from patients with CRC. To identify the miRNAs involved in the regulation of CRC cell proliferation, invasion and metastasis, we examined and compared the gene expression profiles of whole CRC tissues and adjacent non-cancerous mucosa obtained from surgical resections using an miRNA microarray platform (whole genome expression microarrays as described in the Materials and methods). Of the human miRNAs on the array, a variety of miRNAs exhibited a significant difference in expression between the cancerous and non-cancerous samples. Among these miRNAs, 5 were upregulated and 16 were downregulated by >1.6-fold, between the 2 groups (Fig. 1A and Table I). In particular, miR-145 exhibited the most significant difference in expression, with a log2 reduction of 3.43 in the cancer tissues compared with the controls. By using *in silico* analsyis and searching online microRNA databases, we identified NAIP as a potential target of miR-145. Furthermore, we found that NAIP was also a possible shared target of two other significantly downregulated miRNAs, miR-1 and miR-451.

Microarray validation by RT-qPCR of miR-145, miR-1 and miR-451 in CRC tissues. To validate the miRNA microarray data and examine the expression patterns of the focused miRNAs and the target gene, we measured the expression of miR-145, miR-1 and miR-451 in an additional 40 CRC tissue samples by RT-qPCR. These were quantified using the ΔΔCt method together with the previous 20 samples. One of the tested samples was selected as the calibrator, and the relative expression value of this sample was set as 1. As shown in Fig. 1B, we found that the basal expression levels of miR-145, miR-1 and miR-451 were significantly decreased in the tumor tissues as compared with the adjacent non-cancerous control tissues.

Expression patterns of NAIP in cancerous and non-cancerous tissues. To determine the clinical significance of the target gene of miR-145 and miR-1, we measured the NAIP mRNA and protein levels in 40 pairs of matched CRC specimens by qPCR and western blot analysis. The protein expression of NAIP was significantly upregulated in the CRC tissues compared with the adjacent non-cancerous tissues (Fig. 1C-E).
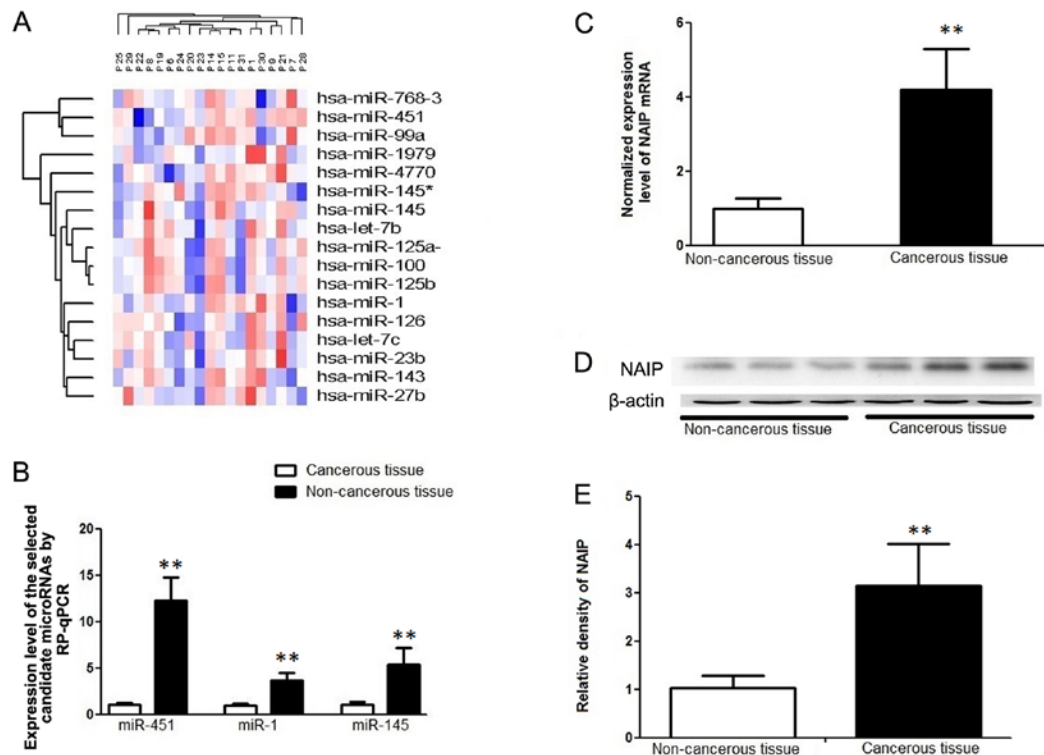
Figure 1. (A) Differentially expressed microRNAs identified using microarray analysis. (B) Expression patterns of miR-1, miR-145 and miR-451. (C) mRNA expression level of NLR family, apoptosis inhibitory protein (NAIP) mRNA in cancerous tissues vs. adjacent non-cancerous controls. (D) Protein expression level of NAIP in cancerous tissues vs. adjacent non-cancerous controls. (E) Densitometric analysis of the western blot analysis results. **P<0.01, compared to non-cancerous tissue.
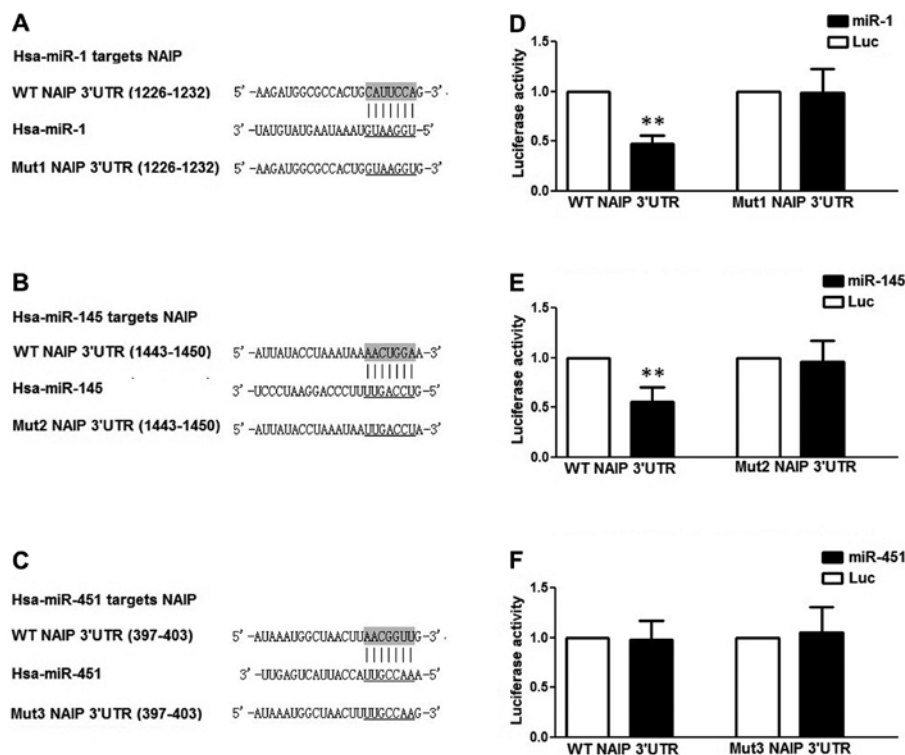

Figure 2. (A) Schematic representation of miR-1 and its binding sequence in the wild-type (WT) or mutant (mut) 3'-UTR of survivin mRNA; (B) schematic representation of miR-145 and its binding sequence in the WT or mut 3'-UTR of survivin mRNA; (C) schematic representation of miR-451 and its binding sequence in the WT or mut 3'-UTR of survivin mRNA; (D) analysis of luciferase activity in miR-1 overexpressing colorectal cancer (CRC) cells 48 h after co-transfection with the control *Renilla* luciferase expression construct pRL-TK and firefly luciferase reporter plasmids containing either WT or mut 3'-UTR of NLR family, apoptosis inhibitory protein (NAIP); (E) analysis of luciferase activity in miR-145 overexpressing CRC cells 48 h after co-transfection with the control *Renilla* luciferase expression construct pRL-TK and firefly luciferase reporter plasmids containing either WT or mut 3'-UTR of NAIP; (F) analysis of luciferase activity in miR-451 overexpressing CRC cells 48 h after co-transfection with the control *Renilla* luciferase expression construct pRL-TK and firefly luciferase reporter plasmids containing either WT or mut 3'-UTR of NAIP. **P<0.01, compared to wild-type.
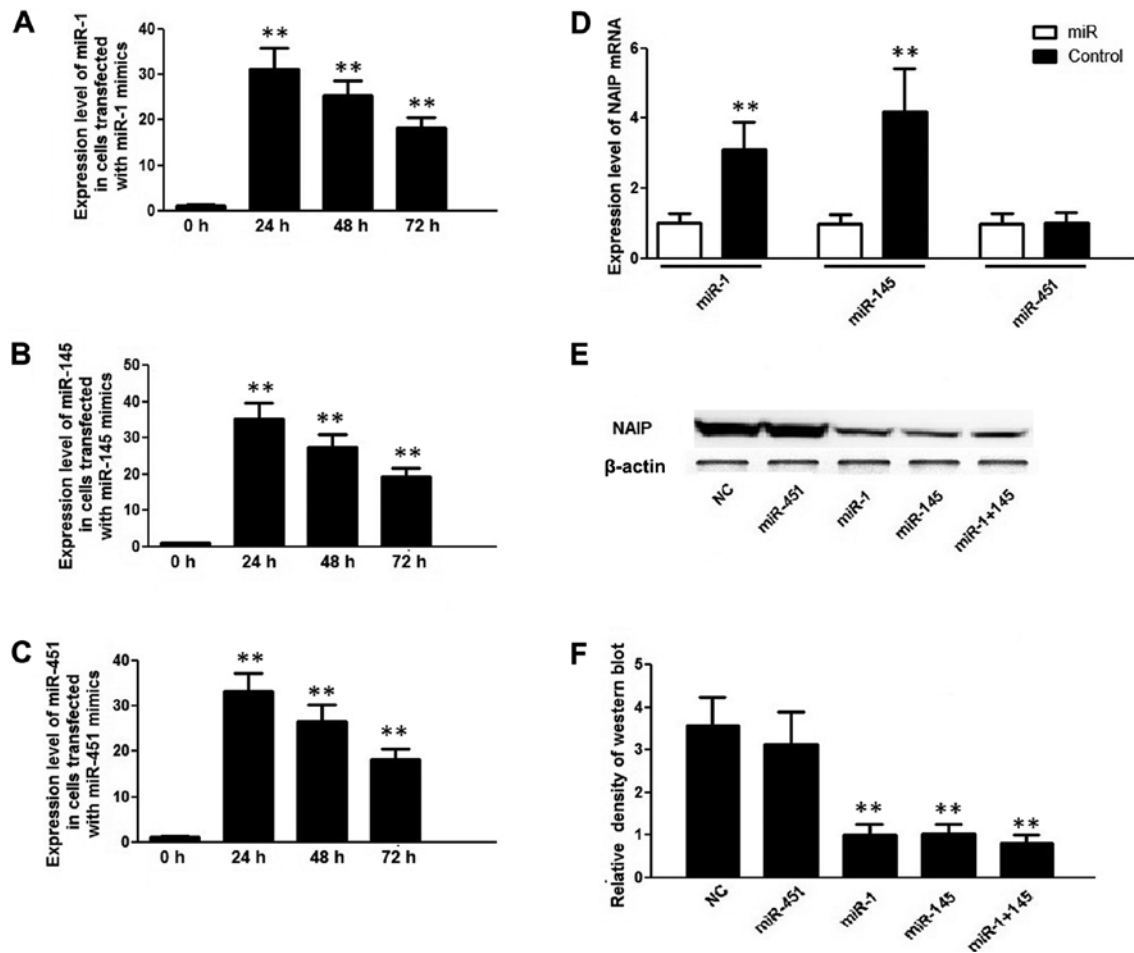
Figure 3. (A) Expression levels of miR-1 24, 48 and 72 h following transfection with miR-1 mimics; (B) expression levels of miR-145 24, 48 and 72 h following transfection with miR-145 mimics; (C) expression levels of miR-451 24, 48 and 72 h following transfection with miR-451 mimics; **P<0.01, compared to the 0 h time point. (D) mRNA expression level of NLR family, apoptosis inhibitory protein (NAIP) following transfection with corresponding microRNAs; (E) protein expression level of NAIP following transfection with corresponding microRNAs, as determined by western blot analysis; (F) relative density of the western blot analysis results. **P<0.01, compared to control. NC, negative control.

NAIP is a shared target of miR-145 and miR-1, but not miR-451 in HCT116 cells. The aforementioned findings indicated that miR-145, miR-1 and miR-451 acted as a cell growth inhibitor in CRC tissues. Therefore, we searched for potential gene targets of miR-145, miR-1 and miR-451, which may contribute to its prometastatic function. We performed *in silico* analysis to search for the potential gene targets of miR-145, miR-1 and miR-451 using the bioinformatics algorithms, TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do). Both algorithms identified NAIP as a potential target gene of miR-145, miR-1 and miR-451 (Fig. 2A-C).

NAIP is a well-known inhibitor of apoptosis that promotes tumor growth. The above findings suggest that the oncogenic function of miR-145, miR-1 and miR-451 in CRC may be attributed to the suppression of NAIP expression. To examine this hypothesis, luciferase reporter assays were performed using luciferase reporters carrying the wild-type NAIP 3'-UTR, and we found that miR-145 and miR-1, but not miR-451, had a significantly decreased activity compared with the controls in the HCT116 cells (Fig. 2D-F).

Furthermore, the HCT116 cells transfected with miR-145, miR-451 and miR-1 mimics exhibited an approximately 30-fold increase in each corresponding miR at 24 h following transfection compared with the controls (0 h) (Fig. 3A-C); however, only miR-1 and miR-145 decreased the mRNA and protein expression of NAIP in the HCT116 cells, and no such effect was observed in the cells transfected with the miR-451 mimic (Fig. 3D-F). These findings corroborate the hypothesis that NAIP expression has an inverse correlation with miR-145 and miR-1 expression, but not with miR-451 expression in CRC cells.

Additionally, miR-1 and miR-145 inhibitors were transfected into the HCT116 cells, but only the miR-1 inhibitor suppressed the expression level of miR-1, and endogenous miR-145 expression was already too low to be further reduced (Fig. 4A and B). As expected, the decrease in the expression of miR-1, caused by the miR-1 inhibitor, led to an increase in the protein and mRNA expression of NAIP in the HCT116 cells (Fig. 4C and D).

Upregulation of miR-1 and miR-145 inhibits HCT116 cell proliferation in vitro. To further examine whether the differentially expressed miRNAs alter the proliferative capacity of the HCT116 cells, miR-145 mimic, miR-1 mimic and miR-145 inhibitor were sequentially transfected into the HCT116 cells, and RT-qPCR was performed to determine miRNA upregulation or downregulation (Figs. 3A, 3B
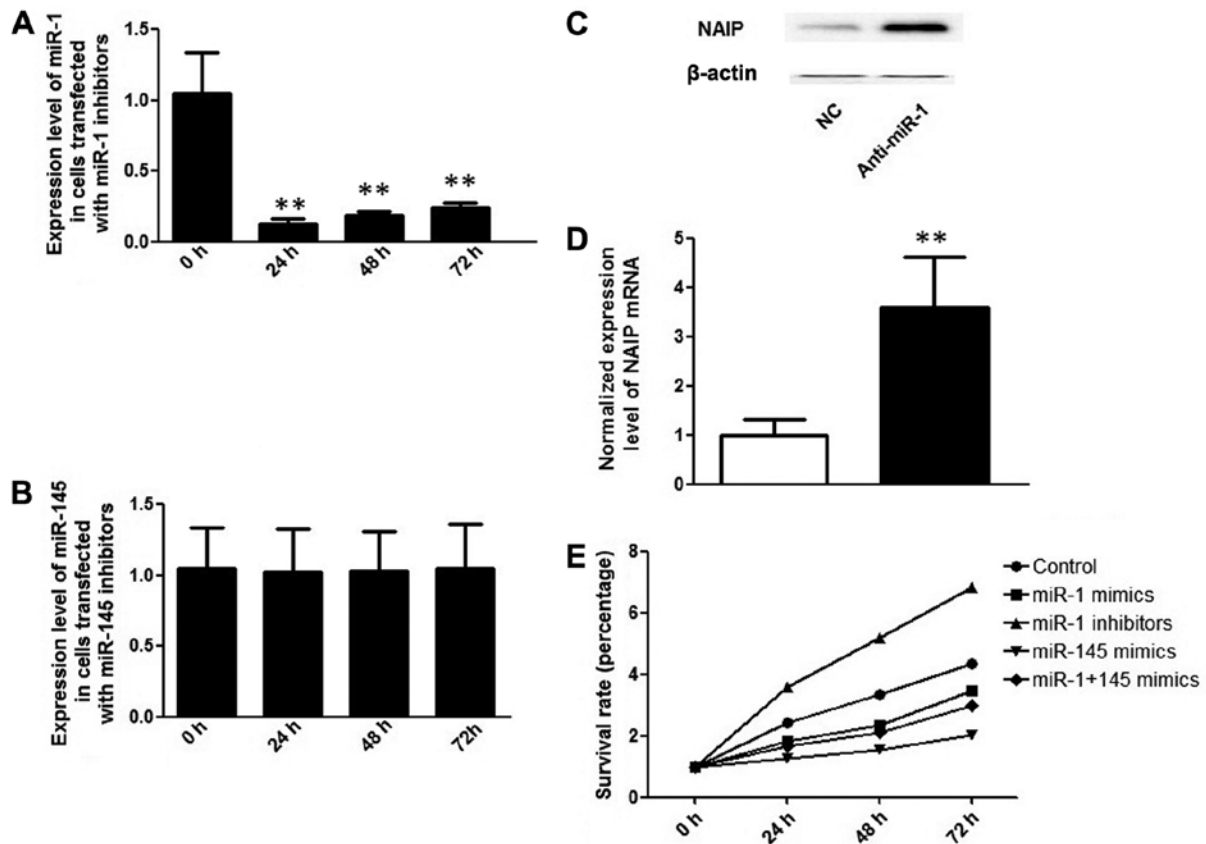
Figure 4. (A) Expression levels of miR-1 24, 48, 72 and 96 h following transfection with miR-1 inhibitors; (B) expression levels of miR-145 24, 48, 72 and 96 h following transfection with miR-145 inhibitors; **P<0.01, compared to the 0 h time point. (C) protein expression level of NLR family, apoptosis inhibitory protein (NAIP) following transfection with miR-1 inhibitors; (D) mRNA expression level of NAIP following transfection with miR-1 inhibitors; **P<0.01, compared to control. (E) Survival rate analysis of colorectal cancer (CRC) cells transfected with miR-1 and/or miR-145 mimics, as determined by MTT assay 24, 48 and 72 h following transfection. NC, negative control.

and 4A). As shown in Fig. 4E, the overexpression of miR-145 and miR-1 significantly suppressed the proliferation of the HCT116 cells, as evidenced by a decrease in the percentage of surviving cells compared with the controls. The introduction of the miR-1 inhibitor evidently suppressed the expression of miR-1, causing an increase in NAIP expression (Fig. 4C) and promoting the proliferation of the cells (Fig. 4E).

Introducing miR-1 and miR-145 significantly induces the apoptosis of HCT116 cells. To elucidate the molecular mechanisms underlying the observed inhibitory effect on CRC cell proliferation of miR-1 and miR-145, we performed flow cytometric analysis using the cells transfected with miR-1 mimic and/or miR-145 mimic. We found that the introduction of miR-1 and miR-145 significantly induced apoptosis both individually and in combination, as shown in Fig. 5A-D, and the apoptotic status of the cells is summarized in Fig. 5E. Our results demonstrate that the inhibitory effect on CRC cell proliferation of miR-1 and miR-145 may be due to their ability to induce the apoptosis of HCT116 cells.

Discussion

CRC is a complex genetic disease which results from abnormalities in gene structure and/or expression (19). The aberrant expression of endogenous miRNAs has been noted in multiple

types of human cancer, such as lung and breast cancer, leukemia and CRC (20), and the deregulated expression of certain miRNAs has been reported to be a marker for early diagnosis, prognosis, as well as the response to treatment in patients with CRC, indicating that miR-145 and miR-1 play a promising role as prognostic or therapeutic biomarkers for patients with CRC (21-23). The balance of oncogenes and tumor suppressor genes plays an essential role in regulating cell proliferation and survival (24,25). Considering the fact that 1/3 protein-coding mRNAs are subjected to miRNA-mediated regulation, the role of miRNAs in the development of malignancies is critical (26). In the present study, using microarray analysis, we identified 21 differentially expressed miRNAs, 5 upregulated and 16 downregulated mRNAs in the colon cancer samples as compared with the adjacent non-cancerous control tissues (Table I).

In non-cancerous tissue, miRNA-mediated control helps to maintain normal cell activities, including growth, differentiation and apoptosis. The deregulation of miRNA expression leads to the abnormal activity of the miRNA target genes, and the consequent upregulation of oncogene(s) and/or the downregulation of tumor suppressor gene(s) results in cells with selective advantages, such as increased proliferation or survival (27). The present study focused on individual miRNAs and their target genes. By using online target prediction tools, we identified NAIP as a possible shared target of three significantly downregulated miRNAs, miR-1, miR-145 and miR-451,
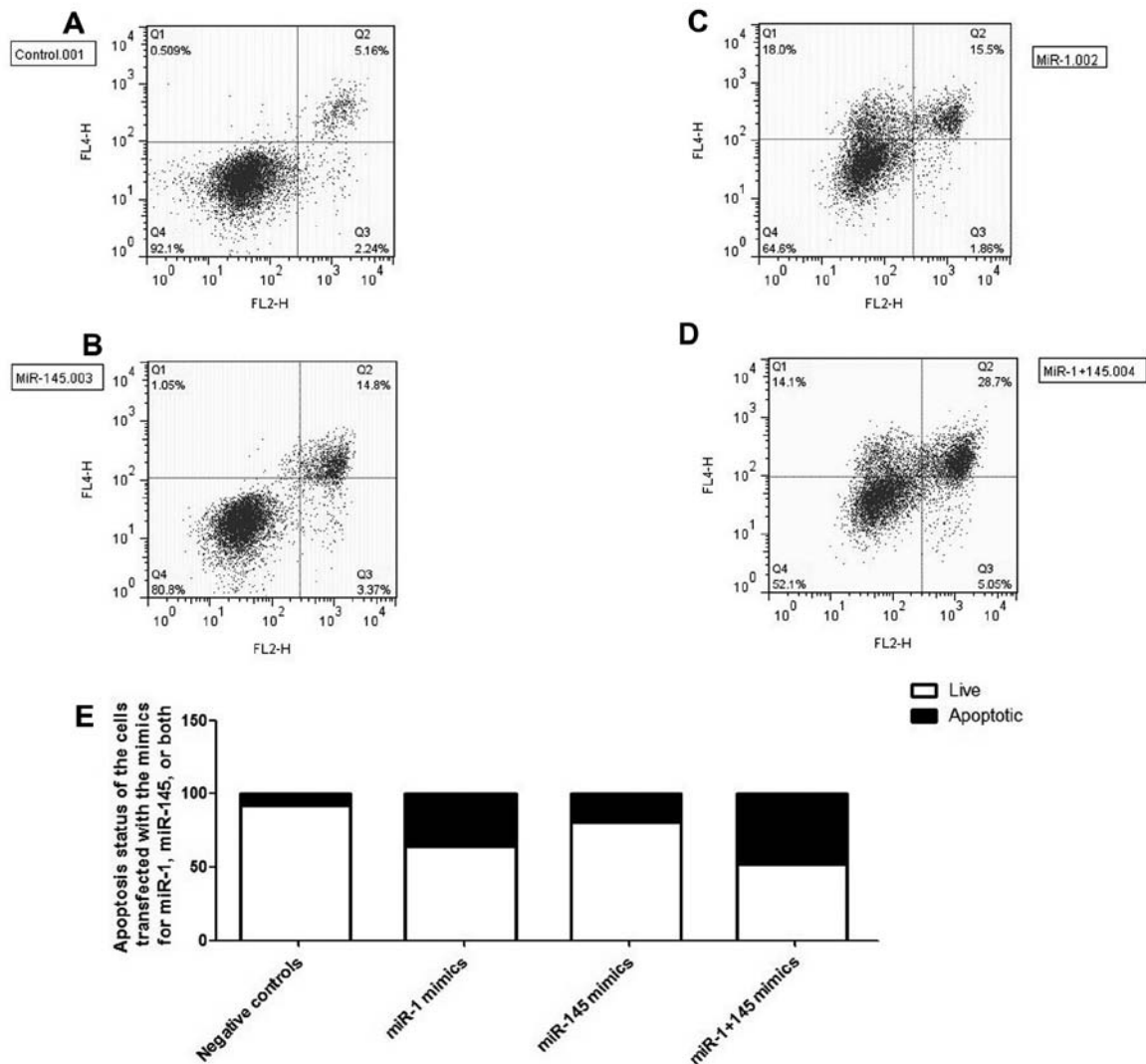
Figure 5. Effect of miR-1 and miR-145 mimics on the apoptotic status of colorectal cancer (CRC) cells. (A) Controls; (B) miR-145; (C) miR-1; (D) miR-1+miR-145; (E) chart of the apoptotic status of cells transfected with negative controls, miR-1 mimics, miR-145 mimics and miR-1 + miR-145 mimics.

and we subsequently demonstrated that NAIP is a valid target of miR-1 and miR-145, but not miR-451, using a luciferase assay (Fig. 2), and this was further confirmed by the observation that the introduction of miR-1 and miR-145 mimics decreased the expression level of NAIP (Fig. 3). miR-145 and miR-1 have been reported to be aberrantly expressed in numerous types of cancer, and their tumor-suppressive roles have been well established. miR-1 is located at 18q11 in the human genome, and is abundantly expressed in the heart and skeletal muscle tissue (28). It has been demonstrated that miR-1 is among the most frequently downregulated miRs in solid human tumors. miR-1 gene expression is reduced in human hepatocellular carcinoma cell lines compared to normal liver cells due to hypermethylation. When the miR-1 gene is hypomethylated, it re-expresses miR-1 and can induce apoptosis (29). In mouse myoblast cells, the mutant MyoD transcription factor downregulates miR-1 expression and inhibits cell apoptosis (30). miR-1 expression has bene shown to be downregulated in human lung cancer tissues and cell lines in comparison to normal human lungs, and miR-1 influences the response of lung cancer cells to anticancer drugs (31). Additionally, miR-1 is downregulated in human bladder cancer (32) and head-and neck squamous cell carcinoma (HNSCC) cells (33). It has been demonstrated that miR-1 acts as a tumor suppressor miRNA by directly targeting various oncogenes, such as prothymosin alpha (PTMA) and transgelin 2 (TAGLN2) (33).

miR-145 is located in a cluster in the 5q32 chromosomal region. Several mRNAs have been demonstrated to be direct targets of miR-145 (34) in colon cancer, and the dysregulation of this miRNA is believed to be responsible for the development of the disease. miR-145, or its analogue, has also been studied in relation to breast (35), leukemia (36), non-small cell lung (37) and prostate cancer (38). One of the best-characterized candidate targets of miR-145 is the oncogene cadherin-17 (CDH17), which is a member of the cadherin superfamily. Cadherins function as calcium-dependent cell adhesion proteins and their dysregulated expression is associated with tumor formation and metastasis (39). It has also been previously demonstrated that CDH17 is involved in proliferation, as it activates Wnt signaling (40). CDH17 was suppressed by >2-fold at the protein

and mRNA level following transfection with miR-145 or its analogue miR-143 (40). Furthermore, it has been shown that chemically unmodified miR-145 complexed with polyethylenimine (PEI) can be used as an efficient and biocompatible strategy for miRNA replacement therapy, which is used in the treatment of colon cancer (41). In the present study, we confirmed the tumor suppressive effect of miR-145 and miR-1 by demonstrating the proliferation-suppressive and apoptosis-inducing effect of the mimics, as well as the proliferation-promoting effect of the inhibitors on CRC cells (Figs. 4 and 5). Moreover, we demonstrated that NAIP, a major anti-apoptotic protein, is an effective target gene of both miR-1 and miR-145 via a luciferase reporter system, and this was further confirmed by the observation that transfection with miR-1 and/or miR-145 suppressed the expression of NAIP.

As its name implies, the inhibitor of apoptosis (IAP) protein family plays an inhibitory role in the regulation of apoptosis and therefore, the overexpression of IAP family members is regarded as an unfavorable feature when diagnosing cancer (42). IAP family proteins are characterized by the presence of one or more baculovirus IAP-repeats (BIR) domains (43), which are zinc-binding folds approximately 70 amino acids long, including three conserved cysteine and one conserved histidine residue (44). The BIR domain is responsible for its ability to inhibit the function of apoptosis executioner caspase-3 and -7, as well as initiator caspase-9 by interacting with IAP-binding motifs (IBMs) in those proteins (43). In addition to the BIR domain, IAPs possess other domains, such as really interesting new gene (RING), caspase activation and recruitment domain (CARD) and nucleotide-binding oligomerization domain (NOD), the presence of which endow IAPs with additional abilities to regulate other accessory biological activities, including cell differentiation, cell cycle progression, cell signal transduction, and immune responses (43). The NAIP/baculoviral IAP repeat-containing protein 1 (BIRC1) gene coding region spans 4,212 nucleotides encoding a 1,403-amino acid protein of the 156 kDa protein, and it contains 3 sequential BIR domains at the N-terminus, which makes it quite typical among other IAPs.

In addition to the BIR domain, NAIP harbors a NOD and a leucine-rich repeat (LRR) (45,46), which makes NAIP function differently from other IAP proteins (43). Due to the presence of the NOD domain and LRR motif, NAIP possesses the properties of both the IAP protein family and the nucleotide-binding domain and leucine-rich repeat containing (NLR) protein family. The NOD domain is essential for oligomerization, a key step in signal transduction, and the LRR domain functions as an intracellular sensor of microbial motifs (47). NAIP, together with some other members of NLR, promotes the synthesis and control of the cytoplasmic multiprotein complex termed 'inflammasome' (47). Considering the significant role that NAIP plays in regulating the inflammatory repsonse as well as the fact that miR-145 is substantially downregulated in patients with ulcerative colitis, it has been postulated that miR-145 may contribute to the pathogenesis of ulcerative colitis and is possibly involved in the mechanisms underlying the malignant transformation of the colonic epithelium into ulcerative colitis (48).

In summary, our data provide evidence that miR-1 and miR-145, which were found to be downregulated in colon carcinoma tissues, are involved in the multistep process of CRC development through the modulation of NAIP expression, which in turn affects cell apoptosis. Furthermore, in CRC cells, miR-1 and miR-145 induce cell death by targeting a major anti-apoptotic protein, NAIP, and contribute to the development of colon cancer. Therefore, both miR-1/miR-145 and NAIP can be regarded as having great potential in colon cancer therapy, and can be targeted by either re-expressing the miRNAs and/or interfering with NAIP function.

Acknowledgements

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