

## Original Article

# Upregulation of microRNA-373 in human colorectal carcinoma by targeting CD44

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**Abstract:** The reduced or increased level of specific microRNA has been found in colon and other cancers, supporting their role in carcinogenesis. Despite the upregulation of microRNA-373 (miR-373) has been reported in a various cancers, its role in colon cancer is totally unknown. Hence, the present study was aimed to investigate the expression and function of miR-373 in colon cancer tissues and cell lines. Quantitative RT-PCR analysis showed that miR-373 expression was significantly increased in colon tumor tissues and colon cancer cell lines as compared with corresponding adjacent normal tissues and cells lines. Furthermore, the expression level of CD44 was negatively correlated with miR-373 levels in colon tumor tissues and cell lines, suggesting that functions of miR-373 are mediated by the suppression of CD44 expression. Loss-of-function and gain-of-function showed that miR-373 reduced cell death and growth suppression of colon cancer HCT-15 cells. Taken together, our results demonstrated that miR-373 expression is upregulated in colon tumor tissues and is a potential oncogenic miRNA, suggesting that miR-373 might be a potential clinical marker and therapeutic target for the prevention of colon cancer.

**Keywords:** Colon cancer, miR-373, CD44, apoptosis, proliferation

## Introduction

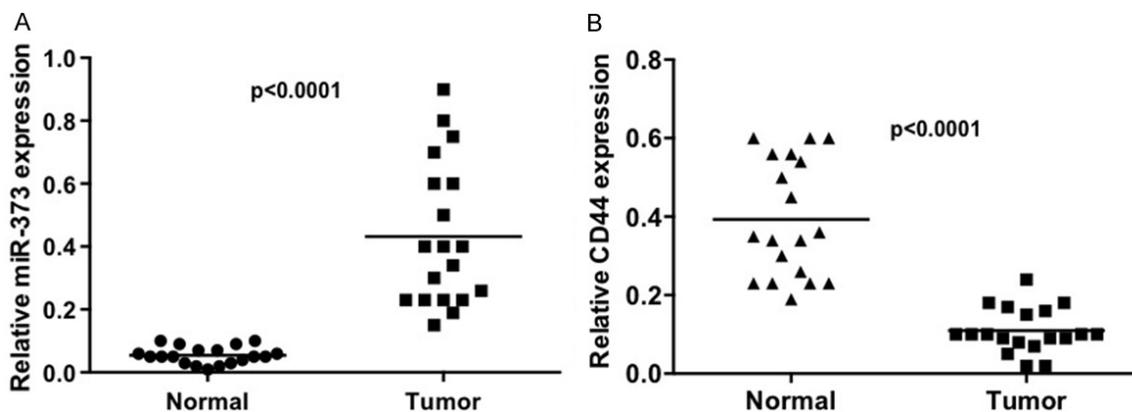
Colorectal cancer is one of the most common malignancies and is the second most common form of cancer-related mortality in the world-wide [1, 2]. Studies have identified the major risk factors for the development of colorectal cancer including age, family history, bowel disease, obesity, alcohol and cigarette smoke, consumption of excess red meat, high-fat diet and low socioeconomic status [3, 4]. The intense invasiveness of the colon cancer and other cancers in patients are the potential cause to death and its critical to understand the genes involved in the progression of colorectal cancer [5]. Therefore, identifying genetic markers and the involved biological signaling pathways that can precisely predict the presence or absence of colon cancer, which is necessary for chemotherapy.

MicroRNAs (miRNAs) are small non-coding RNAs that have been shown to regulate the life span of mRNAs by acting as negative regulators of gene expression through RNA interference pathway [6]. Since miRNAs play a pivotal role in

the control of several biological processes, their dysregulated expression could result in the onset of diverse pathological conditions, including several cancers [7]. MiRNAs expressions are correlated with various cancers and these genes can act as tumor suppressors by negatively regulating oncogenic mRNAs or oncogenes by repressing target mRNAs of tumor suppressor genes [8, 9]. MicroRNA-373 (miR-373) is located on chromosome 19 at position q13.4 and it's an important oncogenic miRNA, which control multiple cancer-related genes and processes [10]. Studies have shown that miR-373 expression is upregulated in several human cancer types, including breast [11], gastric [12], thyroid [13], and bladder cancers [14] by targeting multiple mRNAs such as LATS2 [9, 15], CD44 [16] and ITGA2 [17].

CD44 is a transmembrane glycoprotein, which encodes a cell surface receptor for hyaluronan [18] and is involved in cell-cell and cell-matrix interactions, tumor metastasis and cell migration [19, 20]. Increased expression of CD44 is associated with a favorable outcome, including breast [21] and ovarian cancer [22] patients.

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**Figure 1.** Inverse correlation of miR-373 and CD44 expressions in human colon cancer tissue specimens. RNA was extracted from colon tumor and adjacent normal tissues. (A) miR-373 and (B) CD44 expressions were determined by qRT-PCR, normalized to U6 and GAPDH, respectively, are presented as relative to corresponding normal tissue. The data are expressed as mean  $\pm$  standard deviation. Statistical analyses were performed with Student's t test.  $P < 0.0001$  denotes significant differences between colon tumor tissues and their normal tissues.

Loss of CD44 expression has also been identified in metastatic prostate cancer [23] and colon cancer [24], indicating that CD44 is a metastatic suppressor gene. Studies have reported that CD44 is targeted by several miRNAs, including miR-328, miR-34a and miR-373 [16, 25, 26]. However, the expression and role of miR-373 and its mRNA target, CD44 in human colon cancer tissues and cell lines have not been examined.

In this study, we demonstrated for the first time that miR-373 expression is upregulated in colon cancer tissues and cell lines by directly inhibiting CD44 expression. Subsequent cellular functional studies confirmed that miR-373 inhibited apoptosis and increased cell proliferation in colon cancer cells. Furthermore, miR-373 decreased the expression of CD44 at both mRNA and protein levels. Taken together, these results suggest that inhibition of CD44 by miR-373 stimulates colon cancer proliferation, may be a potential therapeutic target in colon cancer.

### Methods

#### Human colon normal and cancer cell lines

The human normal epithelial colon cell line FHC and cancer cell lines DLD1, HT-29 and HCT-15 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). FHC cells were cultured in DMEM: F12 medium supplemented with 10 ng/ml cholera toxin, 100 ng/mL hydrocortisone and 5 g/ml insulin 10%

FCS, and 1% penicillin/streptomycin. HT-29 cells were maintained in a DMEM medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. All other cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin. All cells were incubated in a humidified atmosphere composed of 5% CO<sub>2</sub> at 37 °C. All chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

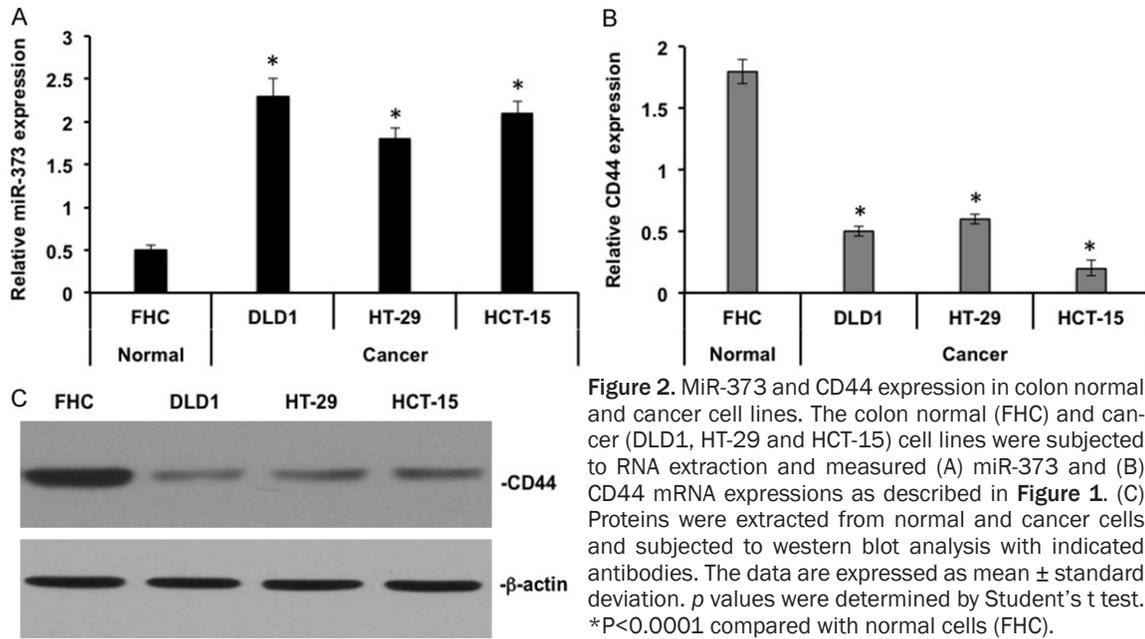
#### Clinical tumor samples

Human colon tumor samples (n=19) along with adjacent normal tissues were immediately collected by biopsy or surgical resection from patients admitted to the Changhai Hospital of Shanghai, Yangpu, Shanghai, China. The patients have not been received pre-operative chemotherapy or radiotherapy before surgery. This study was approved by the Changhai Hospital of Shanghai and informed consent was obtained from patients for this study.

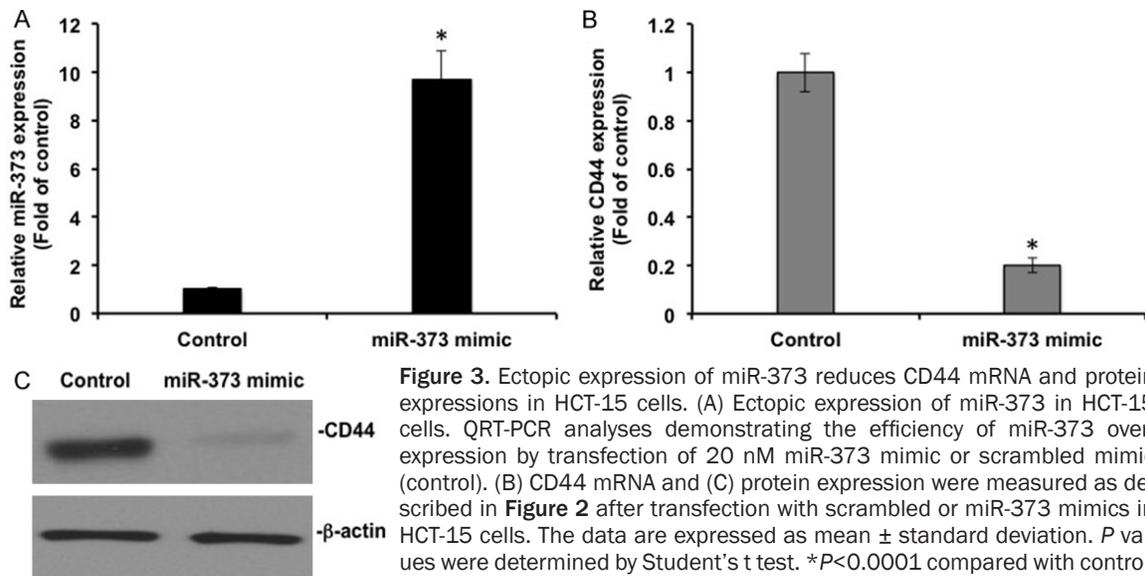
#### Quantitative real time-PCR (qRT-PCR) analysis

Total RNAs were extracted from tumor tissues or cell lines using mirVanamiRNA isolation kit (Ambion, USA). TaqMan probes (Invitrogen) were used to measure miR-373 and CD44 expressions. U6 and GAPDH served as endogenous control, respectively. The PCR reactions were carried in a total of 20  $\mu$ l mixture containing 150 ng of cDNA, 10  $\mu$ l of TaqMan 2X universal PCR master mix and 1  $\mu$ l of probes. The PCR reactions were run on the ABI Prism 7900 Fast

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**Figure 2.** MiR-373 and CD44 expression in colon normal and cancer cell lines. The colon normal (FHC) and cancer (DLD1, HT-29 and HCT-15) cell lines were subjected to RNA extraction and measured (A) miR-373 and (B) CD44 mRNA expressions as described in **Figure 1**. (C) Proteins were extracted from normal and cancer cells and subjected to western blot analysis with indicated antibodies. The data are expressed as mean  $\pm$  standard deviation. *p* values were determined by Student's *t* test. \**P*<0.0001 compared with normal cells (FHC).



**Figure 3.** Ectopic expression of miR-373 reduces CD44 mRNA and protein expressions in HCT-15 cells. (A) Ectopic expression of miR-373 in HCT-15 cells. QRT-PCR analyses demonstrating the efficiency of miR-373 over-expression by transfection of 20 nM miR-373 mimic or scrambled mimic (control). (B) CD44 mRNA and (C) protein expression were measured as described in **Figure 2** after transfection with scrambled or miR-373 mimics in HCT-15 cells. The data are expressed as mean  $\pm$  standard deviation. *P* values were determined by Student's *t* test. \**P*<0.0001 compared with control.

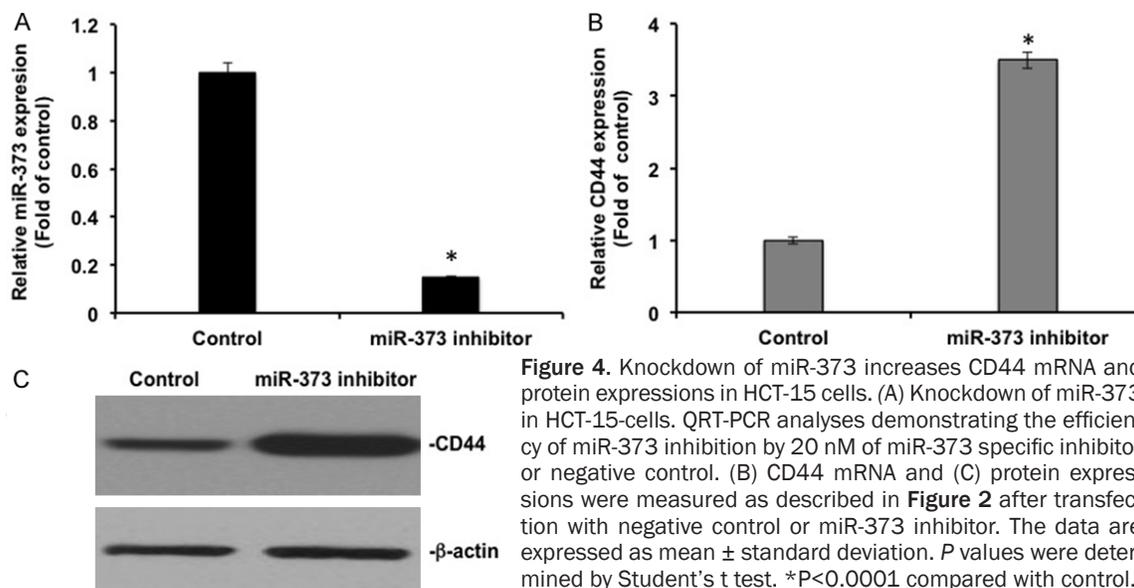
Real-time PCR system for each gene and each sample in triplicate as follows: 95°C for 10 min, 45 cycles of a 15 s denaturing at 95°C, and 1 min annealing at 60°C. SDS 2.1 Software (ABI) was used to calculate miR-373 and CD44 expression levels, normalized to U6 and GAPDH, respectively, and relative to their corresponding controls.

### Western blot analysis

Total proteins were extracted from the cultured cells using a lysis buffer containing 20 mM Tris

(pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM PMSF with protease inhibitor cocktail. The protein concentrations of the supernatant were determined using the Bio-Rad assays. Equal amount of proteins were separated in 10% SDS-PAGE, transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies for overnight at 4°C. The following primary antibodies were used: CD44 (rat monoclonal, 1:1000 dilution, SC-18849) and  $\beta$ -actin (mouse monoclonal, 1:5000 dilution, SC-47778). The corresponding secondary IgG antibodies with alka-

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**Figure 4.** Knockdown of miR-373 increases CD44 mRNA and protein expressions in HCT-15 cells. (A) Knockdown of miR-373 in HCT-15-cells. QRT-PCR analyses demonstrating the efficiency of miR-373 inhibition by 20 nM of miR-373 specific inhibitor or negative control. (B) CD44 mRNA and (C) protein expressions were measured as described in **Figure 2** after transfection with negative control or miR-373 inhibitor. The data are expressed as mean  $\pm$  standard deviation. *P* values were determined by Student's *t* test. \**P*<0.0001 compared with control.

line phosphatase markers were used and incubated for 2 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA. Reacted proteins were detected with chemiluminescence reagent.

### Transfection of miRNA mimics and inhibitors

The synthetic miR-373 mimic (miR-373 mimic) and its scrambled control (control) or miR-373 inhibitor and its negative control (control) were from GenePharma (Shanghai, China). Transfection of either mimic or inhibitor was carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, about  $4 \times 10^5$  cells/well were seeded in a six-well plate. One day after plating, the cells were transfected with 20 nM of either mimic or inhibitor with Lipofectamine (Life Technologies, Grand Island, NY). The media were changed with fresh growth medium after 6 h of transfection. After 48 h of transfection, cells were collected for subsequent analysis.

### Cell proliferation assay

WST-8 Cell Counting Kit-8 was used (CCK-8; Dojindo, Japan) to measure cell proliferation by flow cytometry as described previously [27]. Cells were transfected with either mimic or inhibitor or corresponding negative control. After transfection of 48 h, cells were seeded onto 96-well culture plates at a density of 600 cells/well. After 72 h of incubation, 10  $\mu$ l CCK-8 was added to each well of the 96-well assay

plates and the cells were cultured for 1 h at 37°C in 5% CO<sub>2</sub>. The absorbance at 450 nm was measured using a DNM-9602 microplate reader.

### Apoptosis assay

Apoptotic cells was determined using FITC Annexin V kit (BD Biosciences) according to the manufacturer's instruction as described previously [28]. Briefly, cells were trypsinized and pelleted by centrifugation at 1,000 rpm for 5 min. The cell pellets were then resuspended in binding buffer (500  $\mu$ l), added 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l of propidium iodide (50  $\mu$ g/ml) and incubated at room temperature for 5 min in the dark. Flow cytometry was performed in a FACScan (BD Biosciences). For each data point, triplicate samples were analyzed and the experiments were reproduced three times.

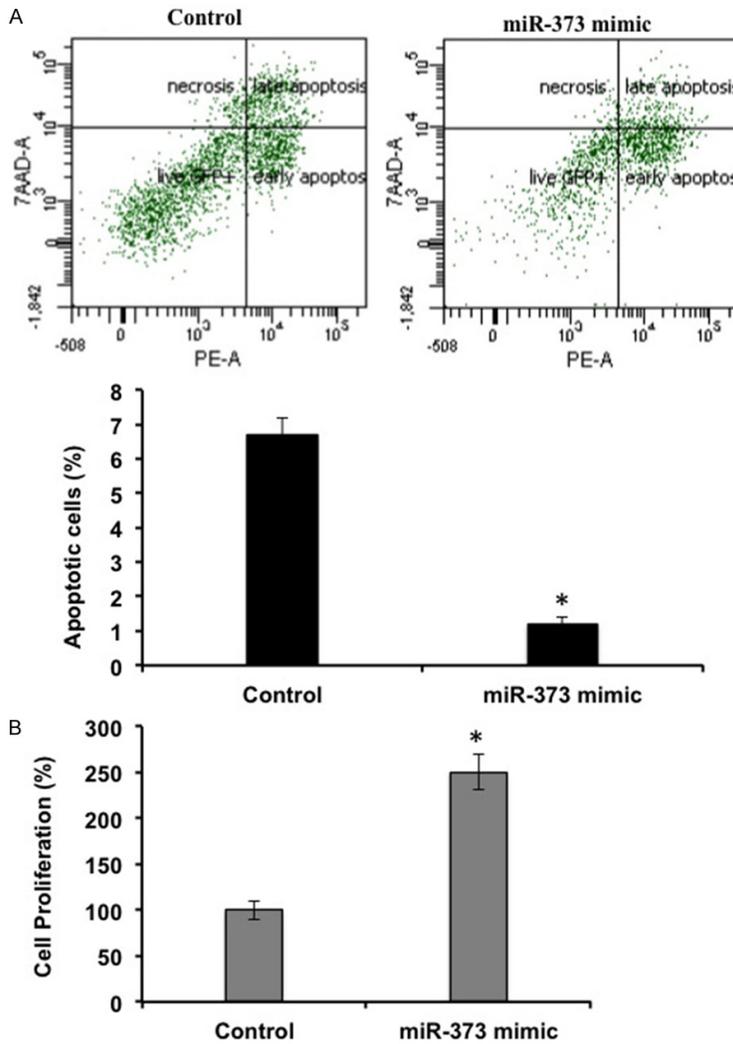
### Statistical analysis

Significant differences were assessed with the Student's *t* test and one-way analysis of variance using SPSS 17.0 software package. Results are presented as mean standard deviation (SD). A *p* value <0.05 was considered as statistically significant.

## Results

### miR-373 is upregulated in human colon cancer tissues and cell lines

To assess the expression of miR-373 in human colon tissues, we evaluated miR-373 expres-



**Figure 5.** Overexpression of miR-373 inhibits apoptosis and increases cell proliferation in HCT-15 cells. A. HCT-15 cells were harvested after 48 h transfection of scrambled or miR-373 mimics and apoptosis was measured by FITC Annexin-V analysis. The representative flow cytometry images and percentage of apoptotic cells are presented. B. Proliferation of HCT-15 cells were measured by WST-8 assay kit after transfection with scrambled or miR-373 mimics and expressed as percentage. Data represent three independent experiments. *P* values were determined by Student's *t* test. \**P*<0.0001 compared with control.

sion in 19 matched pairs of colon cancer and their normal tissues by qRT-PCR. As shown in **Figure 1A**, the expression levels of miR-373 were significantly increased (*P*<0.0001) in tumor tissues as compared with matched normal tissues. Previous study has demonstrated that miR-373 expression is upregulated in breast cancer by targeting CD44. However, the links between miR-373 and CD44 in colon cancer are unknown. Hence, we sought to evaluate the relationship between miR-373 and CD44 in colon cancer; the mRNA expression of CD44

was measured in colon tumor tissues and adjacent normal tissues. CD44 expression was significantly (*P*<0.0001) decreased in tumor tissues when compared with normal tissues (**Figure 1B**).

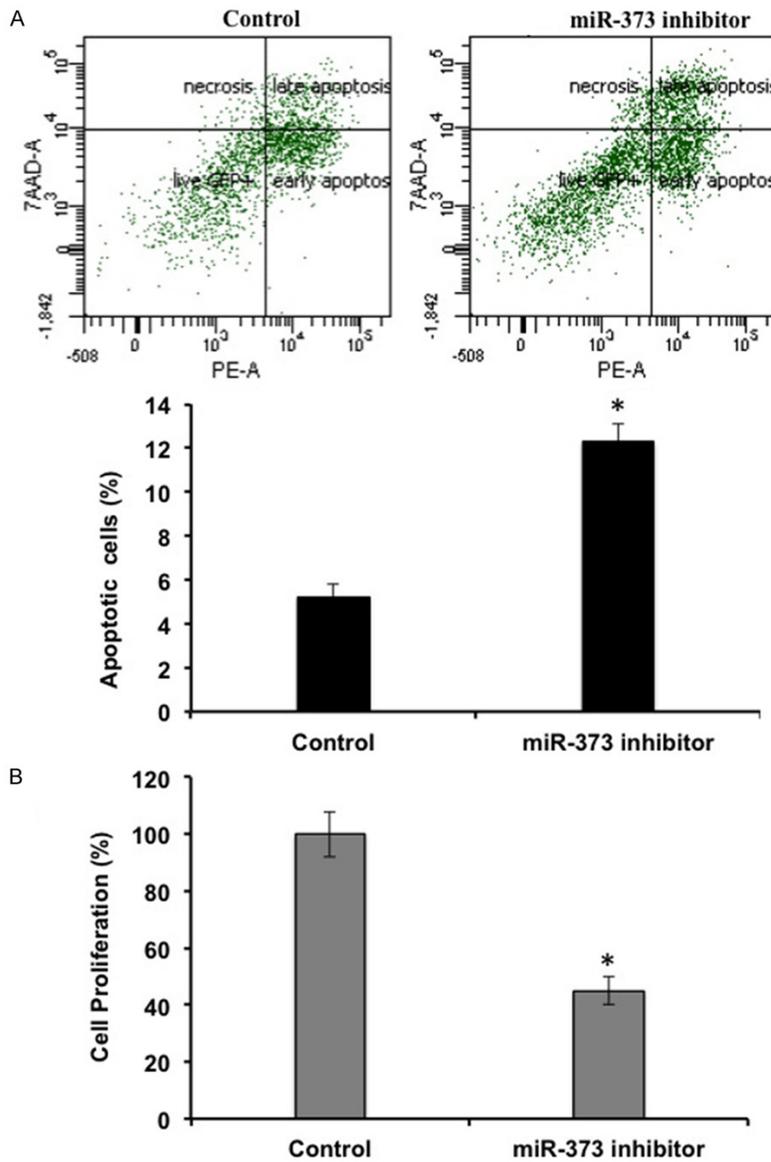
To test whether miR-373 expression is upregulated in different colon cancer cells, we examined miR-373 expression levels in normal and colon cancer cell lines by qRT-PCR. As shown in **Figure 2A**, the expression of miR-373 was significantly (*P*<0.0001) reduced in DLD1, HT-29 and HCT-15 colon cancer cells as compared to FHC colon normal cell line. Subsequently, we examined the CD44 mRNA (**Figure 2B**) and protein (**Figure 2C**) expressions in these cell lines. The CD44 mRNA and protein expressions were considerably decreased in all colon cancer cells, suggesting that CD44 expression is inversely correlated with miR-373 expression in both tumor tissues and cell lines.

*MiR-373 regulates CD44 expression in colon cancer cells*

To see whether ectopic expression of miR-373 affects CD44 expression in colon cancer, we performed overexpression experiments in HCT-15 cells. As shown in **Figure 3A**, cells transfected with miR-373 mimics showed higher expression of

miR-373 in relative to control mimic transfected cells. Overexpression of miR-373 decreased the mRNA and protein levels of CD44 in HCT-15 cell as compared to control cells (**Figure 3B, 3C**).

We then analyzed the loss-of function of miR-373 in HCT-15 cells by inhibiting miR-373 expression with specific inhibitor. As shown in **Figure 4A**, cells transfected with miR-373 inhibitor showed lower expression of miR-373 in relative to control inhibitor transfected cells.



**Figure 6.** Inhibition of miR-373 induces apoptosis and decreases cell proliferation in HCT-15 cells. A. After 48 h transfection of miR-373 inhibitor in HCT-15 cells, apoptosis was measured by FITC Annexin-V analysis. The representative flow cytometry images and percentage of apoptotic cells are presented. B. HCT-15 cells were transfected with miR-373 inhibitor as described in panel A. Cell proliferation was measured by WST-8 assay kit and expressed as percentage. Data represent three independent experiments. *P* values were determined by Student's *t* test. \**P*<0.0001 compared with control.

As we expected, CD44 mRNA and protein expressions were markedly increased in miR-373 knockdown cells (Figure 4B, 4C), indicating that CD44 is a direct target for miR-373.

*MiR-373 modulates apoptosis and cell proliferation of colon cancer cells*

Apoptosis and WST-8 cell proliferation assays were performed to analysis the effects of miR-

373 on HCT-15 cell death and proliferation. The miR-373 mimic transfected cells showed a significant decrease (*P*<0.0001) of apoptotic cells and concomitantly increase the cell number as compared with control cells (Figure 5A, 5B).

On the other hand, knock-down of miR-373 by specific inhibitor significantly (*P*< 0.0001) induced cell death and conversely, decreased HCT-15 cell proliferation, comparing to negative control inhibitor (Figure 6A, 6B). Collectively, these results suggest that miR-373 exhibits a positive role on the proliferation of colon cancer.

**Discussion**

Accumulation of cells with well documented genetic and epigenetic changes can progresses colon cancer from a benign polyp to a malignant adenocarcinoma. Specific genetic changes are starting to inform therapeutic approaches and guide treatment decisions. The most research on colon cancer has been focused on genetic and epigenetic changes in coding genes for their role in colon cancer initiation and progression. The global expression of miRNAs is deregulated in most cancer types. Recent findings revealed that miRNA deregulation in human cancers

occur by multiple mechanisms, including transcriptional deregulation, epigenetic alterations, dysfunction of key proteins in miRNA biogenesis pathway, mutation and DNA copy number abnormalities. Studies have suggested that miRNA expression would be downregulated in human tumors relative to normal tissues, and other studies reported a tumor-specific mixed pattern of downregulation and upregulation of

miRNA genes [29]. Several studies have reported that many miRNAs, including let-7, miR-9, 21, 34, 143, 145, 342 and 345 play important roles in the colon cancer senescence, proliferation, invasion and metastasis by regulating different genes (Reviewed in [30]). MiR-373 is localized in chromosome 19 at position q13.4. MiR-373 is overexpressed in many cancer types, however its expression and function has not been studied in colon cancer. Thus, the aim of this study was to examine the miR-373 expression in colon cancer tissues might lead to the discovery of novel biomarkers for this lethal disease.

In the present study, we demonstrated for the first time that miR-373 was upregulated in colon cancer tissues and cell lines in relative to adjacent normal tissues and colon normal cell lines. Interestingly, CD44, a direct target of miR-373, expression was significantly decreased in both cancer tissues and cell lines. Subsequent loss-of-function and gain-of-function showed that miR-373 decreased cell death and increased cell proliferation by altering CD44 expression. These results suggested that miR-373 has an oncogenic role by targeting tumor suppressor gene CD44, may contribute to the progression and metastasis of colon cancer.

Several *in vitro* studies demonstrated that the upregulation of miR-373 enhanced cell proliferation of human liver carcinoma [31] and testicular germ cell [15]. However, other study reported that miR-373 expression is downregulated in cholangiocarcinoma and is associated with poor cell differentiation, advanced clinical stage, and shorter survival [32]. These controversial results suggest that miR-373 expression is perhaps tumor specific and highly dependent on its targets in different cancers. Multiple studies reported that tumor suppressors, including LATS2, PPP6C, DKK1, TNFAIP1 and TP53INP1 are direct target of miR-373 (Reviewed in [33]), indicating that miR-373 promotes cell proliferation and tumor progression. Consistent with majority of previous reports as oncogenic miR-373, our data from qRT-PCR analysis exhibited that miR-373 is upregulated in all colon tumor tissues, suggesting that miR-373 may act as oncogene in colon cancer progression.

Studies have been reported that increased expression of CD44 is associated with a favor-

able outcome, including breast cancer [21] and ovarian cancer [22]. In addition, CD44 expression is downregulated in metastatic prostate cancer [23] and colon cancer [24], indicating that CD44 is a metastatic suppressor gene. In this study, we showed that CD44 was downregulated in colon cancer tissues and cells as compared to normal adjacent tissues. The expression of CD44 was negatively correlated with miR-373 levels. Therefore, the downregulation of CD44 expression by miR-373 may accelerate the progression of colon cancer.

In conclusion, miR-373 is frequently increased in colon cancer tissues and cell lines and is a potential oncogenic miRNA. Thus, our findings suggest that miR-373 might be a potential clinical marker and therapeutic target for the prevention of colon cancer. However, further studies are needed to investigate the molecular mechanisms by which miR-373 promotes colon cancer through the downregulation of CD44.

### Disclosure of conflict of interest

None

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