MicroRNA-93 inhibits tumor growth and early relapse of human colorectal cancer by affecting genes involved in the cell cycle

I-Ping Yang1,2, Hsiang-Lin Tsai3,4,5, Ming-Feng Hou4,5, Ku-Chung Chen1, Pei-Chien Tsai1, Szu-Wei Huang1, Wen-Wen Chou1, Jaw-Yuan Wang1,4,6, and Suh-Hang Hank Juo1,6,7

1Department of Medical Genetics, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, 2Department of Surgery, College of Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, 3Graduate Institute of Medical Genetics, College of Medicine, Kaohsiung Medical University and 4Cancer Center and Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

Purpose: Colorectal cancer (CRC) is associated with high recurrence and mortality. Because deregulation of microRNAs is associated with CRC development and recurrence, the expression levels of miRNAs can be a simple and reliable biomarker to detect postoperative early relapse, thereby helping physicians to treat high-risk patients more efficiently.

Experimental design: We used microRNA arrays and observed that miR93 had substantially different expression levels in early (recurrence within 12 months after surgery) and non-early relapse CRC patients. The replication study, which included 35 early relapse and 42 non-early relapse subjects, further confirmed overexpression of microRNA-93 in non-early relapse samples. The in vitro and in vivo effects of microRNA-93 were investigated by examining cell proliferation, migration and invasion, as well as cell cycle, target-gene expression and xenograft in null mice.

Results: Cellular studies showed that the overexpression of microRNA-93 inhibited colon cancer cell proliferation and migration but not invasion. The cell cycle studies also revealed that microRNA-93 caused an accumulation of the G2 population. However, miR-93 could not induce cell apoptosis or necrosis. Functional studies showed that microRNA-93 could suppress CCNB1 protein expression leading to cell cycle arrest in the G2 phase. Moreover, microRNA-93 repressed expression of ERBB2, p21 and VEGF, all of which are involved in cell proliferation. MicroRNA-93 also suppressed tumor growth in null mice.

Conclusions: This study showed that microRNA-93 can inhibit tumorigenesis and reduce the recurrence of CRC; these findings may have potential clinical applications for predicting the recurrence of CRC.

Introduction

A mature microRNA (miR) is a non-coding small RNA that contains approximately 20 nucleotides and can post-transcriptionally regulate the expression of several target genes by directly binding to their 3’ untranslated regions (3’-UTR) (1). miRs have been suggested to play a role in the development of cancers, including carcinogenesis, progression and recurrence (2,3). The expression levels of miRs can be either upregulated or downregulated in carcinogenesis and cancer progression (1,4,5). As a consequence, miRs have the potential to serve as biomarkers for cancer detection and prognostic prediction (2,3,6,7). Colorectal cancer (CRC) is a significant public health problem. Each year, there are nearly 1 million newly diagnosed cases of CRC worldwide and approximately half a million deaths (8). For many decades, the depth of tumor invasion, regional lymph node involvement and the presence or absence of distant metastasis have been used as major prognostic factors in CRC patients, in accordance with the American Joint Commission on Cancer/International Union Against Cancer staging (8). Although surgical resection can be highly effective for a localized disease, 25 to 40% of patients develop recurrence after surgery (9); approximately one-third of them develop a local recurrence, whereas the others develop distant metastases (10). The recurrence of CRC is, for the most part, a time-limited phenomenon, where 40–50% of recurrences become apparent within the first year after initial surgical resection (11). It has been shown that the time from the initial treatment to recurrence is strongly related to survival; especially in patients within 1 year of their surgical resection (12,13). Continuous efforts have been made to improve the methods of early detection of tumors in order to improve patient prognosis by providing adequate and effective treatments. At the present time, there is no ideal biomarker or indicator to predict early relapse (2,3,14). A simple and reliable biomarker for the detection of postoperative early relapse would help physicians plan more aggressive treatments and also assist patients to arrange their schedules (3,14).

The tumorigenesis of CRC involves multistep genomic changes, including the activation of oncogenes and inactivation of tumor suppressor genes (15). Recent evidence has suggested that deregulated miRs are involved in the pathogenesis of CRC (1,5,16,17). Although several reports have demonstrated that miRs are related to the development of CRC (3,18,19), studies focusing on the association between miRs and early CRC relapse are sparse.

In the present study, we first used miR arrays to compare the miR profiles in the CRC tissue samples of early and non-early recurrence patients. Early relapse was defined as the recurrence of CRC within 12 months of radical surgery. We identified a promising candidate, miR-93, and validated its role by examining more tissue samples. A series of in vitro and in vivo studies were then conducted to validate and illustrate the role of miR-93 in CRC recurrence. The detailed molecular mechanisms and miR-93 target genes were documented in the present study.

Materials and methods

 Patients and tumor samples

Two cohorts of CRC patients were recruited. The first cohort included 77 subjects with primary CRC in UICC stages I–III (35 non-early relapse patients and 42 early relapse patients after radical resection). The development of postoperative recurrent or metastatic lesions was defined as postoperative relapse. Early relapse was defined as local recurrence (tumor growth restricted to the anastomosis or the region of the primary operation) or distant metastasis (distant metastasis or diffuse peritoneal seeding) occurring within 1 year of radical resection (11,12,20,21). The first cohort was used to determine the miRs related to early recurrence. To test whether a candidate miR was differentially expressed between paired tumor and non-tumor colorectal samples in the same subject, we recruited a second cohort comprising 45 CRC patients. To avoid the potential influence of neoadjuvant treatment on miR expression, patients were excluded if they had undergone neoadjuvant treatment with either chemotherapy or radiotherapy before surgery. There was no available information regarding early relapse status for the second cohort. All subjects were unrelated ethnic Chinese residing in Taiwan. Human tissues were quickly frozen in liquid nitrogen after surgical resection. All clinical samples were obtained with informed consent from each subject, and the study protocol was approved by the local institutional review board.

RNA extraction and cDNA preparation

Approximately 100 mg of each tissue was homogenized using a benchtop homogenizer (Polytron PT1600E, Kinematica AG, Lucerne, Switzerland).
in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA, including miRNA and mRNA, was purified with Qiagen RNAeasy Columns (Qiagen, Hamburg, Germany). For the miRNA array, cDNA synthesis of miRs was performed using Megaplex Reverse Transcription Human Pool A and Pool B (Applied Biosystems, CA, USA). For the individual miRNA assays, cDNA of each miR was synthesized with a unique primer (Applied Biosystems) by using 20 ng of the total RNA. For the miRNA quantitative assay, cDNAs were synthesized from 1 μg of total RNA with random hexamer primers by using Reverse Transcriptase (Applied Biosystems).

Tissue samples from three patients with primary CRC (one non–early relapse and two early relapse) were screened using miR array (Applied Biosystems) containing 667 human miRs and mammalian U6b as the internal control to identify differentially expressed miRs between early and non–early relapse CRC. Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) was performed using the Applied Biosystems 7900HT Real-Time PCR System, and default thermal-cycling conditions by ABI 7900 Sequence detection System version 2.2 (22).

Assay for individual miR

The TaqMan miR RT–qPCR assay (Applied Biosystems) was used to quantify the level of each candidate miR. The relative expression level of the miR was normalized to that of an internal control, U6b, by using the equation of \( \Delta \Delta C_{\text{t}} \), where \( \Delta C_{\text{t}} = C_{\text{mir-93}} - C_{\text{U6b}} \). The mean and standard deviation values of \( \Delta \Delta C_{\text{t}} \) were calculated. We found miR-93 to be the most promising candidate and consequently our further studies focused only on miR-93.

Target prediction

To investigate the biological functions of the miR-93, its target genes were searched by using several miR target prediction programs (22), TargetScan Human (http://targetscan.org), miRDB (http://mirdb.org/miRDB/), miRWalk (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/) and miRanda (http://www.microrna.org/microrna/home.do). Pathway analyses of miR93 target genes were conducted by using the KEGG pathway program (http://www.genome.jp/kegg/kegg2.html) and MetaCore Analytical suite (GeneGo). The ‘Enrichment Analysis’ was used to summarize the possible pathways where contain miR-93 target genes.

Cell culture

The human colonic carcinoma cell lines Caco2 and HCT116 (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco-BRL) and 100 U/ml of penicillin, as described previously (23). The CRC cell line LoVo (ATCC, Manassas, VA, USA) was cultured in F12K medium (Gibco-BRL) supplemented with 10% fetal calf serum (Gibco-BRL) and 100 U/ml of penicillin. The cells were maintained at 37°C in an atmosphere of 5% CO2.

Construction of miR-93 overexpressing constructs

The pCDH vector (System Biosciences, Mountain View, CA, USA) was used as a miR-93 overexpression system to assess the functional consequences of overexpressed miR-93. We constructed the pCDH-miR-93 plasmid by inserting the miR-93 PCR product into the multiple cloning sites. The sequences of these primers for miR-93 were TCTGGAATTCACCTTCACTGAGGCGGGTAGTTGTGA and CTGGGGGCGGCGGGGAGAGAGAGCAGACCATTTGAGAC. The forward primers were extended at the 5’ end to include the GAATTC sequence (these were used to create EcoRI and NotI restriction sites, respectively). The constructs were confirmed by direct DNA sequencing.

Establishment of stable clone

The Caco2 cells (5 × 104) were seeded and transfected with 400 ng of the constructs (either the negative scrambled pCDH vector or the pCDH-miR-93 plasmid) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The stably transfected Caco2 cells containing the pCDH-NC (negative control) or the pCDH-miR-93 plasmid were selected for over 4 weeks by using the standard culture media supplemented with additional 12 μg/ml puromycin (Sigma-Aldrich, St Louis, USA). Confirmation of stable transfection of the plasmids was obtained using the mir qRT–PCR assay.

Analysis of cell proliferation

Cell proliferation was determined by using the WST-1 method (Roche Diagnostics, Indianapolis, IN, USA). Cells were seeded in a 96-well plate and incubated for 22 h and further incubated with 1/10 volume of WST-1 reagent and incubated for another 2 h at 37°C before the absorbance at 450 nm was quantified using a spectrophotometer.
electrophoresis gels, transferred to PVDF membranes and blocked in 0.1% Tween 20 and 5% skim milk protein in Tris-buffered saline. Proteins were probed with primary antibodies overnight at 4 °C. The membrane was washed and visualized with horseradish peroxidase-conjugated secondary antibodies and western blotting substrate (Millipore, Billerica, MA, USA) before exposed to ECL x-ray medical film (Konica Minolta, Mississauga, ON, Canada) to visualize the band. The intensity of each band was determined with HINSTALL software (Bio-Rad Discovery Series), and the intensities of GAPDH bands were used to determine equal protein loading.

**Antibodies**

- Anti-GAPDH was from Sigma–Aldrich; anti-cyclin B1, anti-p21, anti-ERBB2 and Alexa Fluor 594 conjugated donkey anti-rabbit IgG were from Cell Signaling Technology; horseradish peroxidase-conjugated goat secondary antibody was from Promega.

**Immunofluorescence staining**

Immunostaining was performed according to the manufacturer’s instructions (Cell Signaling). The cells were fixed for 20 min with cold methanol solution, washed in cold phosphate-buffered saline and saturated in blocking solution (3% bovine serum albumin in Tris-buffered saline) for 1 h. After being permeabilized with 0.3% Triton X-100 for 5 min, cells were incubated with the primary antibody for 1 h. After washing, the sections were incubated with Alexa Fluor 594 conjugated donkey anti-rabbit IgG, and the nuclei were stained with DAPI (Cell Signaling). For immunostaining in the Caco2 cell, the sections were imaged on a Fluoview FV1000 confocal microscope (Olympus, Hamburg, Germany).

**Statistical analysis**

The continuous variables are represented as mean ± standard deviation values, and the dichotomous variables are represented as number and percentage values. Analysis of covariance was performed using the value of less than 0.05 was considered statistically significant. The statistical power was calculated using the online DSS Research Statistical Power Calculator, on the basis of the following: an alpha of 0.05, 42 early recurrence subjects and 35 non-early recurrence subjects, and the empirical expression levels in each group.

**Results**

**Demographic data**

The characteristics of the first cohort of 77 independent CRC patients (35 non-early relapse and 42 early relapse) are summarized in Table I. The mean age (year) of the patients was 66.4 with the range from 24 to 86 years. The status of early recurrence in this cohort is shown in Table II. The early relapse patients had more advanced stages than the non-early relapse patients ($P < 0.003$; Table II), but their age and sex distributions were not significantly different. The second cohort of 45 patients was used to compare miR-93 expression levels between paired tumor and non-tumor colorectal samples from the same individual. The detailed information of the second cohort is summarized in Supplementary Table 2 is available at Carcinogenesis Online. However, there is no recurrence information for the second cohort.

**miR-93 in the paired samples**

Using the samples from the second cohort, we found that miR-93 expression level was 1.66-fold higher (paired $t$-test $P = 0.03$) in second
45 tumor tissues than in their paired normal counterpart colorectal tissues. Among these paired samples, 62.22% (28/45) of the CRC samples showed higher miR-93 levels than in the adjacent normal tissue (Figure 1C). The expression level of miR-93 was higher in all CRC tumor tissue types than in the normal colorectal samples (Figure 1D). However, the highest levels of miR-93 in the non-early relapse samples were associated with an earlier UICC stage of CRC, implicating miR-93 as a tumor suppression marker.
Overexpression of miR-93 influences cell proliferation and cell cycle
To examine the potential role of miR-93 in tumorigenesis, we evaluated the effect of miR-93 on the growth of Caco2 cells. We established a Caco2 stable clone (OmiR-93) that overexpresses miR-93: the mean value of log_{10}(2−ΔCt) of miR-93 expression was 2.979, which was 7.98-fold higher than the miR-93 expression in the negative control (NC) clone. As shown in Figure 2A, the proliferation rate for OmiR-93 was only 60% of the rate for NC at 24 h ($P = 0.012$). Since some of the predicted miR-93 target genes are related to cell cycle and cell growth, we assessed whether miR-93 can influence the cell cycle by using the flow cytometry. Our data showed a significantly increased accumulation of a G2 population for OmiR-93 (19.8% in OmiR-93 versus 14.7% in NC; $P = 0.013$; Figure 2B). On the basis of annexin V-FITC/PI analysis, overexpression of miR-93 did not induce cell apoptosis or necrosis (Supplementary Fig. 1A is available at Carcinogenesis Online). These findings suggest that overexpression of miR-93 inhibits the growth of colon cancer cells by arresting cells in the G2 phase without causing apoptosis or necrosis. The top 10 predicted pathways where miR-93 target genes are enriched are associated with the cell cycle and cell development (Supplementary Table 3 is available at Carcinogenesis Online).

Effects of miR-93 overexpression on cell migration
The transwell assay (Figure 2C) indicated that OmiR-93 displayed 63.3% slower migration than NC ($P < 0.0001$). Migration inhibition was also shown by wounding healing analysis. The gap distances of NC and OmiR-93 at 24 h were 0.43 and 0.64 mm, respectively, and at 48 h were 0.08 and 0.34 mm, respectively. These findings suggest that the migration ability in OmiR-93 was reduced to 65% of that in NC. The above results consistently show dramatically decreased migration ability when miR-93 is overexpressed (Figure 2E).

Effects of miR-93 overexpression on tumor cell invasion
The matrigel invasion assay showed no significant difference of invasion ability between the OmiR-93 and NC clones. As shown in the

![Fig. 2](https://academic.oup.com/carcin/article-abstract/33/8/1522/2463706) Overexpression of mir-93 in Caco2 cells affects cell proliferation and migration but not cell invasion. (A) miR-93 suppresses tumor-cell proliferation, according to the WST-1 assay ($P = 0.012$). OmiR-93 indicates the stable clone that overexpresses miR-93, and NC indicates the NC stable clone. (B) Overexpression of miR-93 causes significant accumulation of cells in the G2 phase ($P = 0.013$). Black: G0 phase; light gray: S phase; dark gray: G2 phase. (C) Overexpression of miR-93 suppresses tumor-cell migration, according to the Transwell assay ($P < 0.0001$). (D) Overexpression of miR-93 does not affect tumor-cell invasion ($P = 0.825$). (E) Overexpression of miR-93 in cells decreases their migration capability, as indicated by a wider gap at 24 and 48 h. The photographs of tumor-cell migration were obtained during the wounding healing assay.
Figure 2D, the finding suggests that overexpression of miR-93 does not affect the cell invasion ability ($P = 0.825$).

Effect of overexpression of miR-93 in nude mice
To further validate the role of miR-93 in tumorigenesis, we evaluated the effect of miR-93 overexpression on tumor growth in vivo.

The OmiR-93 and NC clones with scrambled pCDH-NC (NC) were injected subcutaneously to allow tumor growth in nude mice. The tumors became palpable 7 days after inoculation and were allowed to grow until the end of third week (Figure 3A and C). Mice that received OmiR-93 cells had significantly smaller ($P = 0.005$) cancer lumps than those that received NC cells (Supplementary Table 4 is available at Carcinogenesis Online; Figure 3B and C). This in vivo

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Effects of miR-93 on tumor growth in an animal model (in vivo) and mRNA expression in vitro. OmiR-93 is the stable clone overexpressing miR-93, and NC is the control stable clone. (A) Photographs of null mice subcutaneously injected with OmiR-93 cells ($n = 4$, upper panel) or with NC cells ($n = 4$, lower panel) were taken 21 days postinjection. (B) The tumor lumps were smaller in the OmiR-93 group (upper panel) than in the NC group (lower panel) after 21 days. (C) The tumor (cm$^3$) growth curve for OmiR-93 (---: gray) and NC cells (----: black) over 21 days ($P = 0.005$). (D) The mRNA expression levels of ERBB2, p21, CCNB1 and VEGF, as determined by qPCR. Compared to NC (gray), OmiR-93 (black) has significantly lower mRNA levels of ERBB2 (27.9%), P21 (26.8%) and VEGF (55.5%) but not of CCNB1 (93.39%). (E) Western blot analysis shows that injection of OmiR-93 cells leads to lower protein levels of miR-93 target genes than that observed in NC. CCNB1 protein can be downregulated by overexpressed miR-93, suggesting that miR-93 only inhibits CCNB1 translation and does not degrade CCNB1 mRNA.
result provided additional support to indicate that overexpression of miR-93 results in a reduction of tumor-cell proliferation in experimental animals.

The effects of miR-93 are mediated by CCNB1, ERBB2, P21 and VEGF. The abovementioned experiments showed that miR-93 suppressed cell proliferation and migration in Caco2 cells and arrested cancer cells in the G2 phase. Next, we tried to identify miR-93 target genes in order to elucidate the anti-oncogenic effect of miR-93. Several miR-93 target genes were predicted by bioinformatic analysis, including cell cycle–related CCNB1 and p21 and ERBB2 and VEGF, which are relevant to cell migration, proliferation and angiogenesis. The mRNA levels of ERBB2 (P < 0.0001), p21 (P = 0.0002) and VEGF (P = 0.025) were significantly lower in the OmiR-93 cells than in the NC clone (Figure 3D). Although CCNB1 mRNA expression levels were not different between two types of Caco2 clones (P = 0.167, Figure 3D), CCNB1 protein levels were prominently reduced by miR-93 (Figure 3E). These results suggest that miR-93 may post-transcriptionally inhibit CCNB1 mRNA translation but does not degrade CCNB1 mRNA.

Since p21 and VEGF have been experimentally shown to be miR-93 target genes (26,27), we used the luciferase reporter assay to confirm CCNB1 and ERBB2 as direct targets of miR-93. The pLuc-CCNB1 and pLuc-ERBB2 plasmid constructs were generated, and both contained the same putative miR-93 binding sequence (CACCUUU) (Figure 4A and B). Transfected different concentrations of miR-93 mimic (25, 50 and 75 nM) caused a dose-dependent suppression in both wild-type 3′-UTRs of CCNB1 and ERBB2 and downregulate their expression levels (Figure 4D and E). Transfected different concentrations of miR-93 mimic (25, 50 and 75 nM) caused a dose-dependent suppression in both wild-type 3′-UTRs of CCNB1 and ERBB2. On the contrary, miR-93 mimic did not have dose-dependent suppression on the mutant-type 3′-UTRs (Supplementary Fig. 1B and C). Luciferase activity of the vector carrying the wild-type 3′-UTR was significantly downregulated by miR-93, but it was not the case for the vector carrying the mutated 3′-UTR. Therefore, these reporter assays indicated that miR-93 can directly target the 3′-UTRs of CCNB1 and ERBB2 and downregulate their expression levels (Supplementary Fig. 1B and C). Luciferase activity of the vector carrying the wild-type 3′-UTR was significantly downregulated by miR-93, but it was not the case for the vector carrying the mutated 3′-UTR. Therefore, these reporter assays indicated that miR-93 can directly target the 3′-UTRs of CCNB1 and ERBB2 and downregulate their expression levels (Figure 4D and E). Transfected different concentrations of miR-93 mimic (25, 50 and 75 nM) caused a dose-dependent suppression in both wild-type 3′-UTRs of CCNB1 and ERBB2. On the contrary, miR-93 mimic did not have dose-dependent suppression on the mutant-type 3′-UTRs (Supplementary Fig. 1B and C). Western blotting showed decreased protein levels of CCNB1, ERBB2 and p21 (Figure 3E). In addition, by immunofluorescence staining with anti-CCNB1 antibodies, we observed that miR-93 could downregulate intracellular CCNB1 levels in the Caco2 cells, especially in the nucleus (Supplementary Fig. 1D is available at Carcinogenesis Online).

Discussion

In the present study, we used miR profiling to identify miR-93 as a potential biomarker for early and non-early relapse CRC. By analyzing more CRC patients, we confirmed a significant decrease of miR-93 in the early relapse subjects. Our data showed that lower levels of miR-93 were significantly associated with more advanced stages of CRC. Through a series of in vitro studies, we showed that miR-93 inhibits cancer-cell proliferation and migration, without influencing apoptosis, necrosis or cell invasion. Overexpression of miR-93 can arrest the cell cycle in the G2 phase, leading to inhibition of cell proliferation. In nude mice, colon cancer cells overexpressing miR-93 displayed a significantly lower growth rate than the control cancer cells. The present study also identified two additional miR-93 target genes ERBB2 and CCNB1. In addition, our studies showed that miR-93 can influence p21 and VEGF; this finding is consistent with that of previous studies (26–29). Although our initial sample size for microarray screening was too small to reach any statistical power, our follow-up results from cellular, animal and human studies consistently supported the inhibitory role of miR-93 in CRC progression and early relapse.

The above studies offer a biological plausibility to support the role of miR-93 in suppressing CRC recurrence. We demonstrated that miR-93 can downregulate p21, ERBB2 and CCNB1. Both p21 and CCNB1 are well-known genes involved in the cell cycle (27,28). In the cell cycle, G1/S transition is inhibited by p21 and G2/M transition is regulated by the cyclin-dependent kinase 1 and CCNB1 complex. Overexpression of miR-93 decreases p21 levels, which, in turn, promotes cells into the S phase for duplication of chromosomes (27); however, overexpression of miR-93 also downregulates CCNB1, consequently suppressing cells into the M phase. When both p21 and CCNB1 are suppressed by miR-93, the cell population in the G2 phase increases; as a result, cells do not divide, and the proliferation rate subsequently decreases. Recently Petrocca et al. reported that miR-93 can suppress p21 but does not significantly increase the proliferation rate or colony formation efficiency in gastric cancer cell lines (27). We found a decrease of cell proliferation that can be explained by inhibition of both p21 and CCNB1. Although p21 has been considered as a tumor suppressor protein, previous studies have reported that cytoplasmic p21 has oncosogenesis/antiapoptotic functions (30,31) leading to a poor clinical prognosis in patients with breast cancer (32). Consequently, a lower miR-93 expression level in early relapse CRC patients implies overexpressed p21 and poor prognosis.

ERBB2 is also known as HER2/neu. Overexpression of the ERBB2 and VEGF genes has been previously reported in cancer patients (33). VEGF is involved in the ERBB2 signaling pathway and can be upregulated by ERBB2 (18,33). We have shown that overexpression of miR-93 can inhibit the mRNA expressions of ERBB2 and VEGF. VEGF has been considered one of the most important angiogenesis factors, and it plays an important role in tumor development, growth and metastasis (34,35). miR-93 has recently been identified as a novel negative regulator of VEGF (26). In several types of tumors, vascular endothelial growth factor receptor can be expressed on the tumor cell surface (36–38). A low level of miR-93 can result in overexpressed VEGF; therefore, it not only stimulates angiogenesis for tumor growth but also acts along with vascular endothelial growth factor receptor on the tumor-cell surfaces as an autocrine growth factor to enhance tumor growth (38). However, Fang et al. recently showed that miR-93 promotes cell growth and angiogenesis in human glioblastoma patients by suppressing integrin-ß8 expression (39). Integrin-ß8 is a tissue-specific protein that is mainly in the placenta, kidney, brain, ovary and uterus (40) and is involved in cell proliferation (41). However, the expression of integrin-ß8 is very low in normal colon tissue and in colon cancer cell lines (42). Accordingly, the findings by Fang et al. may not be applicable to colon cancer.

Higher miR-93 levels have been reported in tumor tissues than in normal tissues, including gastric cancer (27,28), serous ovarian carcinoma (43) and hepatocellular carcinoma (44). We also found that miR-93 levels were higher in the CRC tumor tissues than in the paired adjacent normal tissues. However, a lower level of miR-93 was expressed in overexpressed CRC and CRC tumors. Consequent overexpression of miR-93 can inhibit the mRNA expressions of genes involved in tumorigenesis and recurrence.

The contradictory observations that miR-93 levels are higher in tumor tissues than in paired normal tissues but higher miR-93 levels are located on chromosomes 7 and X, respectively, and they seem to exert a similar expression pattern in tumorigenesis and recurrence. Consequently, a lower miR-93 expression level in early relapse CRC patients implies overexpressed p21 and poor prognosis. miR-93 can downregulate p21, ERBB2 and CCNB1. Both p21 and CCNB1 are well-known genes involved in the cell cycle (27,28). In the cell cycle, G1/S transition is inhibited by p21 and G2/M transition is regulated by the cyclin-dependent kinase 1 and CCNB1 complex. Overexpression of miR-93 decreases p21 levels, which, in turn, promotes cells into the S phase for duplication of chromosomes (27); however, overexpression of miR-93 also downregulates CCNB1, consequently suppressing cells into the M phase. When both p21 and CCNB1 are suppressed by miR-93, the cell population in the G2 phase increases; as a result, cells do not divide, and the proliferation rate subsequently decreases. Recently Petrocca et al. reported that miR-93 can suppress p21 but does not significantly increase the proliferation rate or colony formation efficiency in gastric cancer cell lines (27). We found a decrease of cell proliferation that can be explained by inhibition of both p21 and CCNB1. Although p21 has been considered as a tumor suppressor protein, previous studies have reported that cytoplasmic p21 has oncosogenesis/antiapoptotic functions (30,31) leading to a poor clinical prognosis in patients with breast cancer (32). Consequently, a lower miR-93 expression level in early relapse CRC patients implies overexpressed p21 and poor prognosis. ERBB2 is also known as HER2/neu. Overexpression of the ERBB2 and VEGF genes has been previously reported in cancer patients (33). VEGF is involved in the ERBB2 signaling pathway and can be upregulated by ERBB2 (18,33). We have shown that overexpression of miR-93 can inhibit the mRNA expressions of ERBB2 and VEGF. VEGF has been considered one of the most important angiogenesis factors, and it plays an important role in tumor development, growth and metastasis (34,35). miR-93 has recently been identified as a novel negative regulator of VEGF (26). In several types of tumors, vascular endothelial growth factor receptor can be expressed on the tumor cell surface (36–38). A low level of miR-93 can result in overexpressed VEGF; therefore, it not only stimulates angiogenesis for tumor growth but also acts along with vascular endothelial growth factor receptor on the tumor-cell surfaces as an autocrine growth factor to enhance tumor growth (38). However, Fang et al. recently showed that miR-93 promotes cell growth and angiogenesis in human glioblastoma patients by suppressing integrin-ß8 expression (39). Integrin-ß8 is a tissue-specific protein that is mainly in the placenta, kidney, brain, ovary and uterus (40) and is involved in cell proliferation (41). However, the expression of integrin-ß8 is very low in normal colon tissue and in colon cancer cell lines (42). Accordingly, the findings by Fang et al. may not be applicable to colon cancer.
antitumorigenesis and therefore tumor cells show higher miR-93 levels than the adjacent normal cells. Since miR-93 has an anti-CRC effect, lower miR-93 levels are observed in more advanced cases of CRC and are more likely to provoke early relapse. However, more studies are required to prove this hypothesis. Because of the lack of reliable biomarkers to predict the early recurrence of CRC, our findings suggest that miR-93 can be valuable as a novel and convenient biomarker for prediction and post-operation follow-up.

In summary, the present data show that miR-93 has antioncogenic effects, including the inhibition of colon cancer cell proliferation, migration, angiogenesis and even tumor growth. The association between decreased levels of miR-93 and early relapse in CRC patients

MicroRNA-93 inhibits tumor growth and early relapse of human colorectal cancer

Fig. 4. miR-93 binds 3′-UTR of CCNB1 and ERBB2 and affects their protein synthesis. (A) Schematic diagram of 3′-UTR reporter construct. The 3′-UTR of the target genes is inserted in the 3′ end of the Luc gene in the pMIR plasmid for luciferase activity analysis. (B) The putative miR-93 binding sequence was aligned with 3′-UTR of CCNB1 or miR-93 binding site mutation mRNA of CCNB1 3′-UTR. (C) The putative miR-93 binding sequence was aligned with the mRNA of ERBB2 3′-UTR and miR-93 binding site mutation mRNA of ERBB2 3′-UTR. (D) OmiR-93 indicates the stable clone overexpressing miR-93, and NC indicates the control stable clone. PLuc-UTR or PLuc-UTR-Mu (200 ng) transfected to OmiR-93 or NC, separately. PLuc-CCNB1 can express luciferase mRNA with CCNB1 3′-UTR containing the miR-93 binding site, and PLuc-CCNB1-Mu can express luciferase mRNA with CCNB1 3′-UTR containing the mutated miR-93 binding site. Luciferase activity in OmiR-93 with the PLuc-CCNB1 plasmid is significantly lower (66.9%) than that in NC with the PLuc-CCNB1 plasmid, P = 0.005. (E) PLuc-ERBB2 can express luciferase mRNA with ERBB2 3′-UTR containing the miR-93 binding site, and PLuc-ERBB2-Mu can express luciferase mRNA with ERBB2 3′-UTR containing the mutated miR-93 binding site. Similarly, luciferase activity in OmiR-93 with the PLuc-ERBB2 plasmid is significantly lower (48.43%) than that in NC with the PLuc-ERBB2 plasmid, P = 0.003.
indicates that miR-93 is a potential biomarker for identifying high-risk CRC patients after radical resection.

**Supplementary material**

Supplementary Table 1–4 and Figure 1 can be found at http://carcin.oxfordjournals.org/

**Funding**

National Science Council of the Republic of China (NSC 99-2320-B-037-014-MY3); the Kaohsiung Medical University Hospital (KMUH98-804, KMUH100-OR16); Academia Sinica (Biosignature in Colorectal Cancers), Department of Health, Executive Yuan (Excellence Cancer Research Center Grant DOH101-TD-C-111-002).

**Conflict of Interest Statement:** No conflicts of interest were related to the current study.

**References**


Received January 04, 2012; revised April 14, 2012; accepted May 03, 2012