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

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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 microRNAs 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 microRNA-93 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 cycles, 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 inhibited 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 treatment. 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 would 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 ng of each tissue was homogenized using a benchtop homogeniser (Polytron PT1600E, Kinematica AG, Lucerne, Switzerland)
MicroRNA-93 inhibits tumor growth and early relapse of human colorectal cancer

Analysis of cell migration
The cell migration experiment was performed using Transwell polycarbonate membrane inserts (Millipore, GmbH, Schwalbach, Germany) in 24-well plates, according to the manufacturer's instructions. The cells (1 × 10^5) were plated onto 24-well milliﬁcs and allowed to migrate for 24 h at 37°C. The inserts were rinsed in 1× phosphate-buffered saline, and the cells were removed from the membranes with 1% trypsin in cell culture lysis reagent (Promega, Madison, WI, USA). Since the PCDH vector contained green fluorescence protein (GFP), GFP of the stable clone was then quantiﬁed using a spectrophotometer.

Wound healing assay
After the cells formed a monolayer, a wound was created by manual scraping with a 1×20 micropipette tip. The culture medium was then replaced, and the cells were incubated at 37°C. Wound closure was monitored and photographed at various time points (0, 24 and 48 h) under a microscope.

Matrigel invasion assay
The cell invasion ability was assayed using 24-well Transwell permeable supports (BD Biosciences, San Jose, CA, USA) with 8-µm pore polycarbonate membrane inserts. The cells (1 × 10^5) were plated on the upper chamber and allow invasion for 24 h according to the manufacturer’s instructions. The migrating cells were ﬁxed and stained with crystal violet and photographed under the microscope. Three photographs from each of triplicate membranes were taken for each experimental group.

Analysis of cell cycle
After a 36 h incubation, cell cycle was quantiﬁed using propidium iodide (Sigma-Aldrich) staining and subsequently analyzed using a FACScan cytoﬂuorimeter (Becton Dickinson, NJ, USA) with the CellQuest software (BD Biosciences) according to the manufacturer’s instructions.

Analysis of cell apoptosis and necrosis
For apoptosis and necrosis analysis, cells were ﬁxed in cold methanol, washed in cold phosphate-buffered saline and incubated with annexin V-FITC (BD Biokarmining) and PI (Sigma), according to the manufacturer’s instructions (23). The stained cells were subsequently analyzed within 1 h by ﬂow-cytometric analysis with the FACScan cytoﬂuorimeter (Becton Dickinson ) (24).

In vivo animal studies
Four-week-old BALB/c nude mice (body weight of 12.6 to 15.6 g) were purchased from BioLasco Taiwan, Ltd (Taipei, TW, ROC) and maintained in a specific pathogen free environment (certiﬁcate no.: 26-995029). At 6 weeks of age, each nude mouse was injected subcutaneously with 1 × 10^7 Caco2 cells either NC or OmriR-93 (n = 4 per transfected cell line) in the neck area. The tumor diameter was measured daily, and the tumor volume (cm^3) was calculated using the following formula: volume = width × length × height/2 (25). Animals were killed 3 weeks after seeding tumor cells, and the tumors were examined and counted immediately without prior ﬁxation.

miRNA quantitative assay
To quantify miRNA transcripts, RT real-time PCR with SYBR Green (Applied Biosystems) was performed with the paired primers listed in Supplementary Table 1 is available at Carcinogenesis Online.

Construction of reporter plasmids
The pMIR-REPORT™ miRNA Expression Reporter Vector System (pMIR, Applied Biosystems) was used as a reporter system to assess the effect of miR-93 on protein expressions. The reporter plasmids were constructed by inserting the PCR product of 3′-UTR into the multiple cloning sites of pMIR vector (Figure 4A). We also created the mutant plasmids by using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All the primer pairs used are listed in Supplementary Table 1 is available at Carcinogenesis Online.

Transient transfection and luciferase assay
The pMIR constructs (400ng) were transfected into the stable clone Caco2 cells by using Lipofectamine 2000 (Invitrogen). The cells were lysed 24 h after transfection, and luciferase activities were measured using the Luciferase Assay System (Promega) according to the manufacturer’s protocol. We also tested for the miR-93 dose-dependent effect on its target genes by cotransfection pMIR constructs with mir-93 mimic (25, 50 and 75 nM) into the Caco2 cells using Lipofectamine 2000 (Invitrogen), and the luciferase activities were measured. Each experiment was independently repeated thrice, and each sample was studied in duplicate.

Protein isolation and western blotting
Whole proteins were prepared from cell lysates with lysis buffer (Cell Signaling, Boston, MA, USA). Protein lysates were quantiﬁed using a Bio-Rad protein assay, resolved in 10% sodium dodecyl sulfate–polyacrylamide gel...
Table I. Clinicopathologic characteristics of the 77 UICC
cancer patients included as cohort I in the present study

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Gender (male/female)</td>
<td>42 (54.5)/35 (45.5)</td>
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<tr>
<td>Age (&lt;65 year/≥65 year)</td>
<td>28 (36.4)/49 (63.6)</td>
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<tr>
<td>Maximum size (&lt;5 cm/≥5 cm)</td>
<td>43 (55.8)/34 (44.2)</td>
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<tr>
<td>Location (colon/rectum)</td>
<td>50 (64.9)/27 (35.2)</td>
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<tr>
<td>Stage (I/II/III)</td>
<td>10 (13.0)/35 (45.5)/32 (41.7)</td>
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<tr>
<td>Depth of invasion (T1/T2/T3/T4)</td>
<td>56 (72.7)/27 (25.3)</td>
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<tr>
<td>Vascular invasion (no/yes)</td>
<td>10 (13.0)/60 (77.9)</td>
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<tr>
<td>Perineural invasion (no/yes)</td>
<td>57 (74.2)/22 (28.6)</td>
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<tr>
<td>Histology (WD/MD/PD)</td>
<td>5 (6.5)/63 (81.8)/9 (11.7)</td>
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<tr>
<td>Type of tumor (A/M)</td>
<td>68 (88.3)/9 (11.7)</td>
</tr>
<tr>
<td>Early recurrence* (no/yes)</td>
<td>35 (45.5)/42 (54.5)</td>
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*International Union Against Cancer.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Non-Early</th>
<th>Early</th>
<th>P value</th>
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<tr>
<td>Age (68.3±12.3/64.8±13.9)</td>
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<td>Sex (F/M) (17/48.6/18/42.9)</td>
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<td>UICC</td>
<td>Stage I (8/22.9%/2/4.8%)</td>
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<td>Stage II (19/54.3%/16/38.1%)</td>
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<tr>
<td>Stage III (8/22.9%/24/57.1%)</td>
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<tr>
<td>Median time to relapse (2 samplesb/10 m²/4–12 m)</td>
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International Union Against Cancer.

*bTwo of 35 non-early relapse patients had recurrence in 14th and 33rd m after radical resection, and others are still cancer free by March 2012. The median follow-up time for the non-censor subjects was 33 m by March 2012.

"m, month.

Results from microRNA array and follow-up validation

Three patients with primary CRC (one non-early relapse and two early relapses) were screened using the miR array (Applied Biosystems) to identify miRs differentially expressed in early and non-early relapse CRC (data not shown). We identified several differentially expressed miRs between the CRC tumors of early and non-early recurrent patients. Among the candidate miRs, miR-93 showed a 3.5-fold increase in expression level in early relapse samples compared with that in the non-early relapse samples.

To further confirm that miR-93 expression levels were different between early and non-early relapse patients, we examined 77 additional human CRC tumor samples: 35 from the non-early relapse group and 42 from the early relapse group. There was a 6.1-fold decrease in the expression level of miR-93 in the early relapse than in the non-early relapse samples (the mean log2(2−ΔΔCt) was 2.235 in the non-early relapse group and 1.445 in the early relapse group, adjusted P < 0.0001, Figure 1A). If we separated the samples by the cancer stage, miR-93 levels were always higher in the non-early than in the early recurrent subjects (data not shown). On the basis of the miR-93 expression levels of 2.235±0.372 in the non-early relapse samples and 1.445±0.524 in the early relapse samples, our sample size (35 non-early relapse samples and 42 early relapse samples) provided a power of 100% to detect the expression difference. We analyzed the miR-93 expression in different stages of CRC tumor samples and found an association between miR-93 expression with CRC stage (adjusted P = 0.0325, Figure 1B). A higher expression level of miR-93 was associated with an earlier UICC stage of CRC (mean values of log2(2−ΔΔCt) were 2.055, 1.869, and 1.655 for stage I, II and III, respectively). These validation samples justified the usefulness of miR-93 in predicting the recurrence of CRC after surgery and the need for further functional studies. We measured the miR-93 expression levels (expressed as log2(2−ΔΔCt)) in three different CRC cell lines (Caco2, HCT116 and LoVo) to be 1.532, 1.646 and 1.423, respectively. The miR-93 expression level of Caco2 was similar to that in the early relapse group and the stage III group; therefore, the Caco2 cell line was used for further investigations.

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 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 available at Carcinogenesis Online. However, there is no recurrence information for the second cohort.
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.

![Fig. 1. The expression levels of miR-93 are deregulated in human CRC tumors. (A) miR-93 expression levels in 35 non-early and 42 early relapse CRC samples. The relative expression level of miR-93 is represented by log_{10}(2^{-\Delta Ct}), \Delta Ct = (Ct_{miR-93} – Ct_{U6b}), with U6b as the internal control for normalization. The expression level is significantly decreased in the early relapse samples (P < 0.0001, with adjustment for age, sex and stage of CRC tumor). (B) The mean miR-93 expression levels in different stages of CRC samples (stage I: n = 10, stage II: n = 35, stage III: n = 32). There is a monotonic decrease in expression level that occurs with increase in CRC stage (P = 0.0325, with adjustment for age and sex). (C) The relative expression levels of mir-93 detected by qPCR in the 45 paired human subjects with CRC. The relative expression level of miR-93 was calculated as log_{10}(2^{-\Delta Ct}). Light gray: mir-93 expression in the paired normal tissue. Dark gray: mir-93 expression in the paired tumor tissue. (D) Side-by-side comparison of the expression levels of miR-93 in the normal colorectal tissue, CRC tissue, non-early relapse subjects and early-relapse subjects.]
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...
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*

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**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 \((n = 4,\text{ upper panel})\) or with NC cells \((n = 4,\text{ lower panel})\) 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 \((\text{cm}^3)\) growth curve for OmiR-93 \((- - - : \text{gray})\) and NC cells \((----:\text{black})\) 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 (CAC-UUU) (Figures 4A, B 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). Transflecting 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 oncosenescence/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 overexpression 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; this 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-β8 expression (39). Integrin-β8 is a tissuespecific 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-β8 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 associated with a more advanced CRC stage and was significantly related to early recurrence of CRC. The miR-106a levels were higher in the tumor tissues than in the paired normal tissues of CRC patients (19), which was consistent with our finding. Additionally, lower expression levels of miR-106a are associated with more advanced colon carcinoma and a worse survival in colon carcinoma patients (45). Notably, miR-93 and miR-106a belong to the miR-17 family (46) that consists of 14 mature miRNAs located on chromosomes 7, 13 and X in humans. These miRs have different expression levels in different tissue types (47) and even in different developmental stages (48). Although several miRs can share identical seed sequences, they do not necessarily have identical biological functions. For example, miR-93 targets regulators of DNA replication, but miR-106b, miR-106a, miR-20 and miR-17-5p associate with transcripts involved in DNA replication and mitosis (47) (Figure 1). miR-93 and miR-106a are located on chromosomes 7 and X, respectively, and they seem to exert a similar expression pattern in tumorigenesis and recurrence. The contradictory observations that miR-93 levels are higher in CRC tissues than in paired normal tissues but higher miR-93 levels predict a better prognosis may be explained as follows: an increase of miR-93 in a cell with oncogenic changes is a natural response in...
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 plLuc-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.](https://academic.oup.com/carcin/article-abstract/33/8/1522/2463706)
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/

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**References**


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