

Decreased expression of miR-193a-3p is associated with poor prognosis in colorectal cancer

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Abstract. Epithelial-mesenchymal transition (EMT) is an early and key process in the metastatic cascade during the progression of colorectal cancer (CRC). The aim of the present study was to identify deregulated EMT-related microRNAs (miRNAs/miRs) of CRC and assess the effect of differentially expressed miRNAs on the prognosis of patients with CRC. Genome-wide expression profiling of miRNAs was assessed in 3 EMT-negative and 3 EMT-positive CRC tissues. Differentially expressed miRNA was further validated in 90 pairs of CRC and corresponding paracarcinoma tissues using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A total of 6 miRNAs (miR-10a-5p, miR-204-3p, miR-1224-3p, miR-193a-3p, miR-365a-3p and miR-3678-3p) were identified to be differentially expressed between different EMT statuses of CRC tissues. Following validation using RT-qPCR, 3 miRNAs (miR-10a-5p, miR-365a-3p and miR-193a-3p) were selected for subsequent studies. The expression levels of miR-10a-5p, miR-193a-3p and miR-365a-3p were markedly increased compared with levels in corresponding paracarcinoma tissues. Survival analyses revealed that down-regulation of miR-193a-3p was associated with worse prognosis of patients with CRC, particularly in female and

older patients. The results of the present study indicate that miR-193a-3p may be an EMT-related biomarker and serve as a novel prognostic factor for CRC.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide, ranking third among males and second among females (1). In 2012, 1,360,602 (9.7%) novel cases were diagnosed and 693,993 (10.9%) mortalities were attributed to CRC worldwide (1). There have been improvements in the diagnosis and therapy of this disease; however, the majority of CRC patients present with recurrence and metastasis, accompanied by a poor prognosis and a low survival rate (2-4). Invasion and metastasis are the most important causes of CRC treatment failure and mortality, therefore previous research has focused on decreasing invasion and metastasis of CRC.

A phenomenon known as epithelial-mesenchymal transition (EMT) has been identified as an early and key process that is associated with progression and metastasis in multiple epithelial cancer types, including CRC (5). The loss of epithelial markers [including epithelial (E-) cadherin] often occurs during EMT. E-cadherin is an important adhesion molecule in cell-cell adhesion in epithelial cells. E-cadherin-catenin complexes are able to decrease tumor cell invasion and metastasis, and inhibit tumor cell proliferation. Structural changes and aberrant expression of E-cadherin are able to induce cell detachment and migration. Previous studies have identified that E-cadherin may serve as a significant prognostic indicator in cancer (6,7). It has also been identified that the expression level of E-cadherin was significantly decreased in CRC (8,9). Aberrant expression of E-cadherin was considered to be associated with vascular invasion, lymph node metastasis and tumor differentiation (8,9).

MicroRNAs (miRNAs/miRs) are endogenous small RNAs of 18-23 nucleotides that regulate gene expression in plants and animals. A number of miRNAs have been investigated and identified to be involved in CRC (10-14). The role of miRNAs in tumor metastasis has been gradually uncovered in previous studies (15,16). However, research into the association between

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E-cadherin and miRNAs, and the potential risk prediction of EMT-associated miRNAs in CRC has been limited. The aim of the present study was to identify differentially expressed miRNAs associated with E-cadherin expression in CRC tissues using microarray analysis. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to verify the results of the microarray analysis. Differentially expressed miRNAs were further evaluated in 90 CRC tissues to investigate the potential association between their expression and clinicopathological parameters and prognosis in CRC.

Materials and methods

Patients. A total of 96 patients with CRC were recruited for the present study. Patients from Taizhou People's Hospital (Taizhou, China) were pathologically diagnosed with CRC and underwent surgical resection between July 2006 and May 2007. Formalin-fixed paraffin-embedded tissues were stored in the Biobank at the National Engineering Center for Biochip at Shanghai (Shanghai, China). Of the 96 cases, 3 E-cadherin-negative and 3 E-cadherin-positive CRC tissues were used for miRNA microarray analysis. A total of 90 patients were used for further analysis on the association between miRNA expression and clinicopathological characteristics. The expression of E-cadherin was investigated using a monoclonal anti-E-cadherin (cat. no. IR05961; 1:100; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) on the Ventana Discovery-Ultra automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions. Furthermore, no patient received chemotherapy or radiotherapy treatment prior to surgery. In addition, 90 matched paracarcinoma tissues were collected at the same time and the sampling point was >5 cm from the tumor margin. The endpoint of the present study was overall survival. All patients provided written informed consent, and the present study was approved by the Committees of Taizhou People's Hospital (Taizhou, China) and the National Engineering Center for Biochip at Shanghai. CRC staging was according to the tumor-node-metastasis (TNM) system as described previously (17).

RNA extraction. The extraction of total RNA from formalin-fixed paraffin-embedded tissues was performed using a RecoverAll™ Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

The samples were deparaffinized by adding 1.0 ml xylene, followed by incubation for 5 min at 50°C, and centrifugation for 5 min at maximum speed. Next, the supernatant was discarded, and the pellet was washed twice with 1.0 ml absolute ethanol for rehydration. The proteins were degraded with 200 μ l digestion buffer and 5 μ l protease, followed by incubation for 15 min at 50°C and for 15 min at 80°C. Subsequently, RNA was isolated by adding 790 μ l buffer from the RecoverAll™ Total Nucleic Acid Isolation kit along with passage through a purification column. The column was then washed twice with a buffer from the kit, and DNase treatment was performed, followed by two additional washing steps. Finally, RNA was eluted in 60 μ l of elution buffer from the kit at room temperature. RNA quantity and purity were determined using

a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

miRNA microarray analysis. miRNA microarray assays were performed using a human miRNA microarray (8x60K; Design ID: 046064) (Agilent Technologies, Inc.) according to the manufacturer's protocol. Microarray assays were performed for 3 E-cadherin-negative and 3 E-cadherin-positive CRC tissues from 6 patients with CRC. Total RNA was dephosphorylated using calf intestinal alkaline phosphatase, denatured with dimethylsulfoxide, and labeled with cyanine 3-conjugated cytidine-5'-phosphate-3'-(6-aminohexyl) phosphate with T4 RNA ligase using a miRNA Complete Labeling and Hybridization kit (Agilent Technologies, Inc.). Probes were hybridized at 55°C for 20 h with rotation. The slides were washed with Gene Expression wash buffer 1 (Gene Expression Wash Pack buffer kit; Agilent Technologies, Inc.) at room temperature for 5 min and subsequently with Gene Expression wash buffer 2 at 37°C for 5 min. Following hybridization and washing, the microarray was scanned using an miRNA scanner (Agilent Technologies, Inc.). Images were captured using Feature Extraction software (version 10.7.3.1; Agilent Technologies, Inc.) and data were analyzed using GeneSpring GX software (version 10.0.2; Agilent Technologies, Inc.). Differences in miRNA expression between E-cadherin-negative and -positive CRC tissues were identified when the fold change of *Caenorhabditis elegans* miR-39-normalized expression values was >2.0 and false discovery rate P-value was <0.05.

RT-qPCR. RNA samples from 3 E-cadherin-negative and 3 E-cadherin-positive CRC tissues were used for RT-qPCR. RT was performed using a miScript Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) in a final volume of 20 μ l containing 6 μ l total RNA, 4 μ l 5X miScript RT buffer and 1 μ l miScript reverse transcriptase mixture as described previously (18). The reaction mixtures were incubated at 37°C for 60 min, 95°C for 5 min and held at 4°C. qPCR was performed on the resulting cDNA using the miScript SYBR Green PCR kit (Qiagen GmbH) using an ABI 7900 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each qPCR was performed in a final volume of 20 μ l containing 1X QuantiTect SYBR Green PCR Master mix (Qiagen, Hilden, Germany), 2 μ l cDNA and 0.5 mM of each primer. The primer sequences are as follows: hsa-miR-10a-5p forward, 5'-TACCCTGTAGATCCGAATTTGTG-3', hsa-miR-1224-3p forward, 5'-CCCCACTCCTCTCTCCTCAG-3', hsa-miR-193a-3p forward, 5'-AATGGCCTACAAAGTCCCAGT-3', hsa-miR-204-3p forward, 5'-GCTGGGAAGGCAAAGGGACGT-3', hsa-miR-365a-3p forward, 5'-TAATGCCCTAAAAATCCTTAT-3' and hsa-miR-3678-3p forward 5'-CTGCAGAGTTTGTACGGA CCGG-3'. The miScript Universal primer was used as reverse primers. The reaction mixtures were incubated at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 70°C for 30 sec. All reactions were performed in triplicate. The expression levels of miRNAs were normalized to the endogenous control U6 small nuclear RNA, and were calculated with the formula $2^{-\Delta\Delta Cq}$ (19). The primer sequence for U6 is as follows: forward, 5'-CTCGCTTCGGCAGCA CA-3' and reverse, 5'-AACGCTTACGAATTTGCGT-3'.

Statistical analyses. All data were analyzed statistically using SPSS software (version 20.0; IBM SPSS, Armonk, NY, USA). Differences between CRC and corresponding paracarcinoma tissues were calculated using Student's t-test and presented as the mean \pm standard deviation. The expression levels of miRNAs were compared using a Mann-Whitney U test. The expression levels of miRNAs were categorized into high and low groups according to the 75th percentile expression levels of miRNAs. A χ^2 test or Fisher's exact test was used to determine the association between miRNA level and clinicopathological characteristics. In order to analyze the effect of miRNA survival of patients with CRC, survival curves were obtained using the Kaplan-Meier estimator method and assessed using a log-rank test. Survival data were also evaluated using Cox's multivariate regression analysis. Hazard ratios and the corresponding 95% confidence intervals were assessed with Cox regression models. All analyses were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miRNA expression profiling of clinical samples. miRNA microarray analysis was used to compare miRNA expression profiles of E-cadherin-negative CRC tissues with E-cadherin-positive tissues (n=6; 3 E-cadherin-positive and 3 E-cadherin-negative CRC tissues). The microarray used for this analysis contained 2,549 human mature miRNAs. A total of 6 miRNAs (miR-10a-5p, miR-1224-3p, miR-193a-3p, miR-204-3p, miR-365a-3p and miR-3678-3p) demonstrated marked differential expression between the two groups. Notably, all 6 miRNAs demonstrated significantly increased expression in E-cadherin-positive CRC tissues ($P < 0.05$, Fig. 1).

To validate the array results, these 6 miRNAs were further verified using RT-qPCR. As presented in Fig. 2, the RT-qPCR results demonstrating increased expression of miR-10a-5p, miR-193a-3p, miR-365a-3p and miR-3678-3p were consistent with the miRNA microarray results.

Expression levels of miRNAs in CRC and corresponding paracarcinoma tissues. As the expression levels of miR-3678-3p in CRC and corresponding paracarcinoma tissues were low ($C_q > 30$), it was excluded from further analysis. The expression levels of miR-10a-5p, miR-193a-3p and miR-365a-3p were examined in 90 pairs of CRC and corresponding paracarcinoma tissues. It was found that miR-10a-5p, miR-193a-3p and miR-365a-3p were upregulated in CRC tissues compared with corresponding paracarcinoma tissues ($P < 0.05$; Fig. 3). In addition, the expression levels of miR-193a-3p in patients with TNM stage I-II were significantly increased compared with those in patients with TNM stage III-IV ($P = 0.032$; Fig. 3). Larger tumors (≥ 6 cm) exhibited increased expression levels of miR-193a-3p compared with smaller tumors (< 6 cm) ($P = 0.012$; Fig. 3).

Association of miRNA expression with clinicopathological characteristics. A total of 90 CRC samples were divided into two groups on the basis of the 75th percentile expression of miR-10a-5p, miR-193a-3p and miR-365a-3p, respectively. No significant association was identified between miRNA

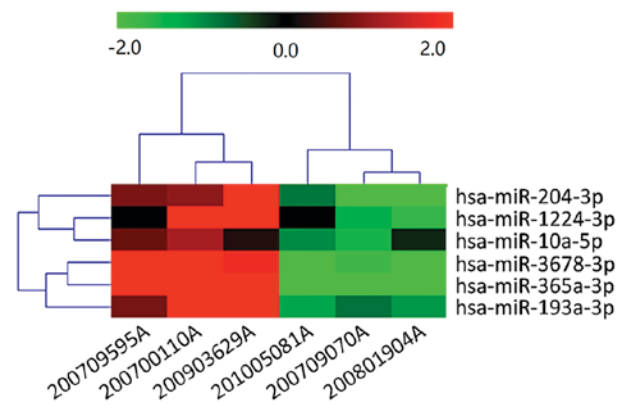


Figure 1. Heatmap of the miRNA transcriptome of through performing microarray analysis in three pairs of E-cadherin-positive (200700110A, 200709595A and 200903629A) and E-cadherin-negative (200709070A, 200801904A and 201005081A) colorectal cancer tissues. miRNA, microRNA; E-cadherin, epithelial cadherin; hsa-miR, human miRNA.

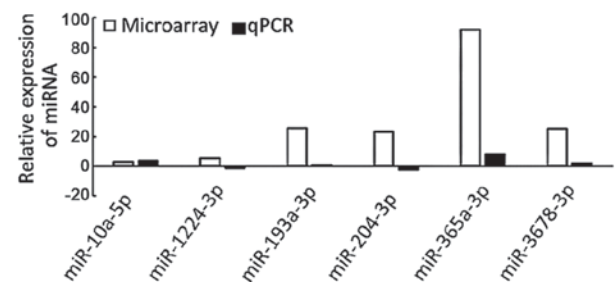


Figure 2. RT-qPCR validation of differentially expressed miRNAs identified in patient colorectal cancer tissues using microarray analysis. miRNA/miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

expression and any clinicopathological characteristic (Table I). Clinicopathological characteristics were not available from all patients.

Survival analyses. Among the 90 patients with CRC, the median survival time was 65 months. The 3-, 5- and 7-year survival rates were 65.6, 51.1 and 44.7%, respectively. To assess the association of miR-10a-5p, miR-193a-3p and miR-365a-3p with the prognosis of patients with CRC, Kaplan-Meier estimator analysis and the log-rank test were used. Patients with low expression of miR-193-3p had a significantly decreased survival time compared with those with high expression of miR-193-3p ($P = 0.012$; Fig. 4A). In univariate analysis, advanced TNM stage [hazard ratio (HR), 1.938; 95% confidence interval (CI), 0.356-1.132; $P = 0.006$] and miR-193a-3p expression levels (HR, 1.250; 95% CI, 0.712-2.196; $P = 0.017$) were associated with worse prognosis of patients with CRC. Multivariate Cox's analyses indicated that TNM stage (HR, 1.730; 95% CI, 1.085-2.759; $P = 0.021$) and miR-193a-3p expression (HR, 2.454; 95% CI, 1.027-5.863; $P = 0.043$) were independent prognosis factors for overall survival rates of patients with CRC (Table II).

Stratified analysis was performed to evaluate the effect of clinicopathological characteristics on the ability of miR-193a-3p expression in predicting the prognosis of patients

Table I. Association between miRNA expression and clinicopathological characteristics.

Characteristic	miR-10a-5p			miR-193a-3p			miR-365a-3p		
	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value
Age, years			0.673			1.00			0.291
≥70	22 (50.0)	25 (55.6)		35 (52.2)	12 (54.5)		26 (59.1)	21 (46.7)	
<70	22 (50.0)	20 (44.4)		32 (47.8)	10 (45.5)		18 (40.9)	24 (53.3)	
Sex			0.399			0.140			0.673
Male	26 (57.8)	21 (46.7)		39 (57.4)	8 (36.4)		25 (55.6)	22 (48.9)	
Female	19 (42.2)	24 (53.3)		29 (42.6)	14 (63.6)		20 (44.4)	23 (51.1)	
Tumor size, cm			0.274			0.071			0.189
≥6	14 (31.8)	20 (45.5)		22 (32.8)	12 (57.1)		14 (31.1)	20 (45.5)	
<6	30 (68.2)	24 (54.5)		45 (67.2)	9 (42.9)		31 (68.9)	23 (53.5)	
Differentiation			0.857			0.309			0.857
Well	1 (2.2)	4 (8.9)		5 (7.4)	0 (0)		2 (4.4)	3 (6.7)	
Moderate	27 (60.0)	22 (48.9)		37 (54.4)	12 (54.5)		25 (55.6)	24 (53.3)	
Poor	17 (37.8)	19 (42.2)		26 (38.2)	10 (45.5)		18 (40.0)	18 (40.0)	
pT category			0.195			0.337			0.853
T1	0 (0)	3 (7.0)		3 (4.5)	0 (0)		1 (2.3)	2 (4.5)	
T2	2 (2.2)	5 (11.6)		4 (6.1)	2 (9.1)		3 (6.8)	3 (6.8)	
T3	40 (88.9)	28 (65.1)		52 (78.8)	16 (72.7)		35 (79.5)	33 (75.1)	
T4	4 (8.9)	7 (16.3)		7 (10.6)	4 (18.2)		5 (11.4)	6 (13.6)	
TNM stage			0.277			0.129			0.515
I and II	20 (44.4)	14 (31.1)		38 (56.7)	17 (77.3)		26 (57.8)	29 (65.9)	
III and IV	25 (55.6)	31 (68.9)		29 (43.3)	5 (22.7)		19 (42.2)	15 (34.1)	

miRNA/miR, microRNA; pT, pathological tumor; TNM, tumor-node-metastasis.

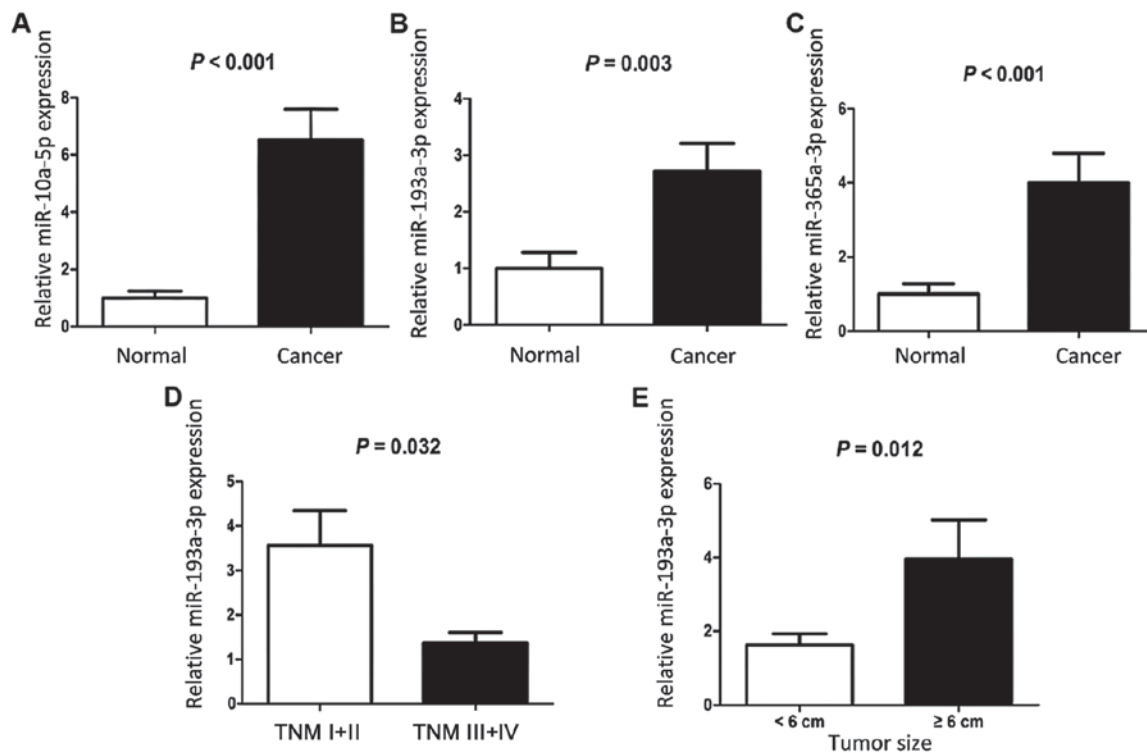


Figure 3. Expression levels of miRNAs in 90 pairs of colorectal cancer and adjacent normal tissues. (A) miR-10a-5p. (B) miR-193a-3p. (C) miR-365a-3p. Expression levels of miR-193a-3p were determined in association with (D) TNM stage and (E) tumor size. miRNA/miR, microRNA; TNM, tumor-node-metastasis.

Table II. Univariate and multivariate analysis of overall survival.

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, ≥ 70 years vs. < 70 years	0.634 (0.356-1.132)	0.123		
Sex, male vs. female	0.784 (0.445-1.382)	0.400		
Tumor size, ≥ 6 cm vs. < 6 cm	1.091 (0.606-1.966)	0.772		
Differentiation, poor vs. well and moderate	1.283 (0.786-2.093)	0.318		
TNM stage, III and IV vs. I and II	1.938 (1.214-3.093)	0.006	1.730 (1.085-2.759)	0.021
pT category, T3+T4 vs. T1+T2	1.548 (0.891-2.691)	0.121		
miR-10a-5p expression, high vs. low	1.126 (0.642-1.975)	0.678		
miR-193a-3p expression, high vs. low	2.846 (1.208-6.705)	0.017	2.454 (1.027-5.863)	0.043
miR-365a-3p expression, high vs. low	1.250 (0.712-2.196)	0.437		

HR, hazard ration; CI, confidence interval; TNM, tumor-node-metastasis; pT, pathological tumor; miR, miRNA.

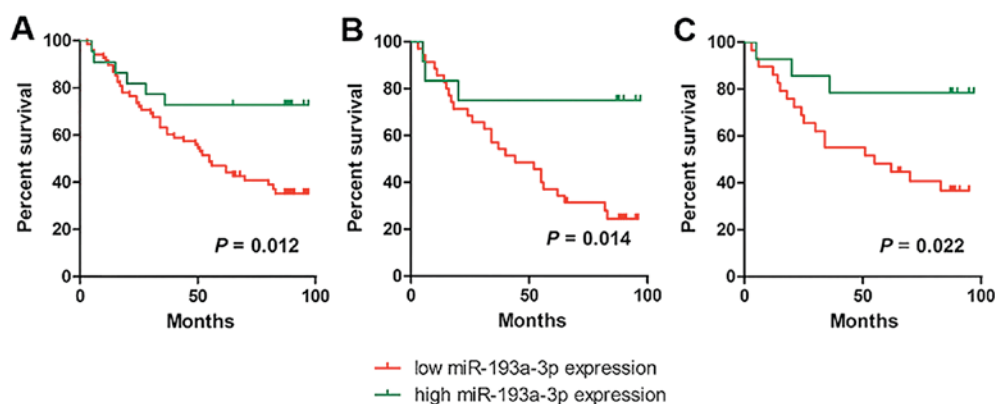


Figure 4. Kaplan-Meier estimator survival curves of overall survival rates in patients with colorectal cancer on the basis of high or low (median) expression levels of miR-193a-3p. (A) All patients. (B) Patients ≤ 70 years. (C) Female patients. miR, microRNA.

with CRC. miR-193-3p was significant in patients with CRC ≥ 70 years (HR, 4.048; 95% CI, 1.215-13.487; $P=0.014$; Fig. 4B) and female patients with CRC (HR, 3.801; 95% CI, 1.115-12.961; $P=0.022$; Fig. 4C).

Discussion

For cancer cells to sustain continuous proliferation, evade growth suppression, invade tissues and form distant metastases, acquisition of certain capabilities is required. In this process, EMT is necessary (20). A key step in EMT noted in various types of cancer of epithelial origin is the loss of E-cadherin expression (21). This has been demonstrated in previous studies of CRC (22-24). Therefore, in order to identify novel biomarkers of CRC, an miRNA microarray assay was used to investigate differentially expressed miRNAs between E-cadherin-positive and -negative CRC tissues. In total, 3 miRNAs (miR-365a-3p, miR-193a-3p and miR-10a-5p) were differentially expressed between E-cadherin-positive and -negative CRC tissues, and were upregulated in CRC tissues compared with paracancerous tissues. Furthermore, expression of miR-193a-3p exhibited a significant decrease in

TNM stages III and IV compared with TNM stages I and II ($P=0.032$; Fig. 3). The survival analysis revealed that down-regulation of miR-193a-3p is associated with worse prognosis of patients with CRC. These results suggest that miR-193a-3p may act as a negative regulator in the development of CRC and that dysregulation of miR-193a-3p may be a novel means of identifying a high risk of developing metastases and requirement of further adjuvant therapies.

Previous studies have demonstrated that a number of miRNAs are associated with the occurrence and development of CRC by affecting cancer cell proliferation and invasion. Nielsen *et al* (25) identified that miR-21 was upregulated in CRC and was associated with TNM stage, and that the overexpression of miR-21 led to a worse prognosis. Schepeler *et al* (26) identified that miR-145 was able to inhibit the proliferation of CRC cells. Furthermore, a decreased level of miR-106 has been identified to predict shortened disease-free survival and overall survival in CRC (27). However, the majority of studies are simply investigating the differential expression of miRNAs between CRC and normal tissues, and investigation of the association between miRNAs and EMT in CRC remains limited. In the present study, miR-193a-3p was

identified to be associated with E-cadherin expression and to be associated with the prognosis of patients with CRC, which was consistent with the role of E-cadherin in CRC prognosis. These results suggest that miR-193a-3p and E-cadherin may be associated in CRC.

Typically, miRNAs are able to regulate post-transcriptional silencing of target mRNAs. Previous studies have suggested that miR-200c regulates E-cadherin expression by inhibiting the expression of E-cadherin repressors including zinc finger E-box-binding homeobox 1/2, through which the ability of cancer cells to migrate and invade neighboring tissues or distant organs is regulated (28-30). Although differentially expressed miRNAs identified by microarray assay did not include miR-200c in the present study, a positive association between the expression of miR-193a-3p and E-cadherin expression was identified. It has been reported previously that the expression level of E-cadherin may be influenced by miR-193a-3p expression: Yu *et al* (31) identified that the expression level of E-cadherin was significantly increased following treatment of lung cancer cells with miR-193a-3p mimic. As the 90 CRC tissues in the present study did not undergo microdissection performed on paraffin-embedded tumor samples, this may partly explain why there was a weak but not statistically significant association between miR-193a-3p expression and TNM stages. The results of the present study indicate that miR-193a-3p regulates E-cadherin expression by acting on E-cadherin repressors. Further studies are warranted to elucidate the association between miR-193a-3p and E-cadherin and to explore the effect of miR-193a-3p on CRC.

The results of the present study provide the first evidence that miR-193a-3p is differentially expressed between E-cadherin-negative and -positive CRC tissues. Furthermore, miR-193a-3p expression is hypothesized to be an independent prognostic biomarker for CRC. However, further extensive studies are required to elucidate the underlying molecular mechanisms of miR-193a-3p in regulating E-cadherin expression and to define its role in the development and progression of CRC.

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