microRNA-499-5p promotes cellular invasion and tumor metastasis in colorectal cancer by targeting FOXO4 and PDCD4

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MicroRNAs (miRNAs) regulate tumor progression and invasion via direct interaction with target messenger RNAs (mRNAs). We defined miRNAs involved in cancer metastasis (metastamirs) using an established *in vitro* colorectal cancer (CRC) model of minimally metastatic cells (SW480 line) from a colon adenocarcinoma primary lesion and highly metastatic cells (SW620 line) from a metastatic lymph node from the same patient 1 year later. We used microarray analysis to identify miRNAs differentially expressed in SW480 and SW620 cells, focusing on miR-499-5p as a novel candidate prometastatic miRNA whose functions in cancer had not been studied. We confirmed increased miR-499-5p levels in highly invasive CRC cell lines and lymph node-positive CRC specimens. Furthermore, enhancing the expression of miR-499-5p promoted CRC cell migration and invasion *in vitro* and lung and liver metastasis *in vivo*, while silencing its expression resulted in reduced migration and invasion. Additionally, we identified FOXO4 and PDCD4 as direct and functional targets of miR-499-5p. Collectively, these findings suggested that miR-499-5p promoted metastasis of CRC cells and may be useful as a new potential therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is one of the three leading global causes of cancer-related death, with approximately 1 020 000 new cases and 530 000 deaths worldwide per year (1,2). Although advances have been made in the treatment of CRC over the last decades with new surgical techniques, radiotherapy and chemotherapy, the overall survival rate of patients with CRC has not shown marked improvement. Metastasis plays a critical role in the poor prognosis, and more than one-third of patients with CRC will ultimately develop metastatic disease (3). Currently, little is known about the exact molecular mechanisms underlying CRC metastasis (4). In general, metastasis results from a complex cascade of biological processes. Understanding the key factors in these processes is crucial to the development and optimal use of novel anticancer therapies.

MicroRNAs (miRNAs) are single-stranded, noncoding RNA molecules of approximately 22 nucleotides that result from the sequential processing of miRNA transcripts by the RNaseIII enzymes Drosha and Dicer (5). miRNAs control gene expression by targeting messenger RNAs (mRNAs) and triggering either translational repression or mRNA degradation (6).

Abbreviations: CRC, colorectal cancer; FOXO, Forkhead box O-class; HIEC, human intestinal epithelial cell line; UTR, untranslated region; miRNA, microRNA; mRNA, messenger RNA; RT-PCR, reverse transcription–polymerase chain reaction; qRT-PCR, quantitative RT-PCR.

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Convincing evidence shows that miRNAs are often aberrantly expressed in human cancer (7–10) and that they can affect key cell biological processes that affect tumor progression, such as migration, invasion, epithelial-to-mesenchymal transition (11,12) and metastasis (13–15). Ma *et al.* (16) demonstrated that miR-10b stimulates invasion and metastasis in breast cancer. Asangani *et al.* (17) found that miR-21 promotes invasion, intravasation and metastasis in CRC. The challenge ahead is to expound intrinsic mechanisms by which miRNAs regulate such processes.

The SW480 and SW620 human cell lines are a validated *in vitro* model for colon cancer that represents the progression from primary to lymph node metastatic stages (i.e. from stage II to stage III). The SW480 colon cancer cell line was obtained from a primary stage II lesion, and the SW620 cell line was established from the same patient a year later from a colon cancer lymph node metastasis (stage III) (18). Thus, the SW480 and SW620 cells have the same genetic background but different metastatic potential (19).

In this study, we identified miRNAs such as miR-499-5p that are involved in metastasis by comparing miRNA expression patterns in the primary carcinoma cell line SW480 and in the corresponding metastatic cell line SW620. We further demonstrated that the upregulation of miR-499-5p in SW620 was associated with the development of metastasis. These results suggest that miR-499-5p is a novel metastasis marker in CRC and might be a potential target for diagnosis and therapy.

Materials and methods

Ethics statement

All experimental procedures were approved by the Institutional Review Board of the Fourth Military Medical University. Written informed consent was obtained for all patient samples. Animal experiments were performed with the approval of the Institutional Committee for Animal Research, in conformity with national guidelines for the care and use of laboratory animals.

Cell culture

The human CRC cell lines SW480, SW620, HT29 and LOVO and the normal human intestinal epithelial cell line (HIEC) were conserved in our own laboratory and were routinely maintained in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 100 U/ml penicillin sodium and 100 mg/ml streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% CO_2. Cells were used in logarithmic growth phase.

miRNA microarrays

Total RNA was extracted from each cell line using the miRVana miRNA Isolation Kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. The quality and quantity of RNA samples were assessed by standard electrophoresis and spectrophotometer methods. miRNA microarray analysis was performed by LC Sciences (Houston, TX). Microarrays used uParaflo microfluidic chip technology (Atactic Technologies, Houston, TX) with optimized RNA hybridization probes with a detection limit of <100 attomoles. Cross-referencing was to the Sanger miRBase release 13.0 (http://www.sanger.ac.uk/Software/Rfam/mirna/) after normalization. The data from the microarray were collected and analyzed in accordance to the MIAME guidelines.

RNA extraction and real-time quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted from cultured cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). For detection of miR-499-5p expression, stem–loop reverse transcription–polymerase chain reaction (RT-PCR) was performed using an All-in-One™ miRNA quantitative RT-PCR (qRT-PCR) Detection Kit (GeneCopoeia, Rockville, MD) according to the manufacturer's instructions. Briefly, the extracted RNA was reverse transcribed in the presence of a poly-A polymerase with an oligo-dT adaptor. Real-time PCR was carried out with SYBR green detection with a forward primer for the mature miRNA sequence and a universal adaptor reverse primer. Relative expression was evaluated by the comparative CT (threshold cycle) method and normalized to the expression of U6 small RNA. Primers for miR-499-5p and U6 were

from GeneCopoeia (HmiRQP0543, HmiRQP9001). For detection of FOXO4 and PDCD4 mRNA, qPCR was performed using TaKaRa SYBR Green PCR Kit (TaKaRa). Glyceraldehyde 3-phosphate dehydrogenase was used to normalize FOXO4 and PDCD4 mRNA expression levels. Primers for FOXO4 and PDCD4 were designed using Primer Express Software (Version 1.5). The primer FOXO4 sequences were (forward) 5′-CTTTCTGAAGACTGGCAG-GAATGTG-3′ and (reverse) 5′-GATCTAGGTCTATGAT CGCGGCAG-3′. The primer PDCD4 sequences were: (forward) 5′-GAT-TAACTGTGCCAACCAGTCCAAAG-3′ and (reverse) 5′-CATCCACCTCC-TCCACATCATACAC-3′. The primer glyceraldehyde 3-phosphate dehydrogenase sequences were: (forward) 5′-AGCCTTCTCCATGGTGGT-GAA-3′ and (reverse) 5′-ATCACCATCTTCCAGGAGCGA-3′. All qRT-PCR reactions were performed in triplicate.

Clinical samples

Samples from 90 patients (50 males and 40 females) who had undergone proctocolectomy with lymph node dissection for CRC at Xijing Hospital between November 2008 and April 2009 were included (Supplementary Table SI is available at *Carcinogenesis* Online). Patients were 31 to 87 years old

Table I. Summary of significantly differentially expressed miRNA in SW620 relative to SW480

Rank	miRNA	Expression in SW620	Mean fold change (SW620/SW480)[a]
1	hsa-miR-499-5p	Up	8.05
2	hsa-miR-9	Up	5.28
3	hsa-miR-125b	Up	4.52
4	hsa-miR-148a	Up	4.18
5	hsa-miR-146a	Up	3.91
6	hsa-miR-9[a]	Up	3.89
7	hsa-miR-100	Up	3.76
8	hsa-miR-424	Up	3.17
9	hsa-miR-1247	Up	3.16
10	hsa-miR-185	Down	−7.13
11	hsa-miR-744	Down	−4.99
12	hsa-miR-193a-5p	Down	−4.15
13	hsa-miR-365	Down	−4.07
14	hsa-miR-141	Down	−4.06
15	hsa-miR-455-3p	Down	−3.88
16	hsa-miR-31	Down	−3.44
17	hsa-miR-203	Down	−3.19
18	hsa-miR-1306	Down	−3.17
19	hsa-miR-483-5p	Down	−3.08
20	hsa-miR-155	Down	−3.07
21	hsa-miR-200c	Down	−3.06

[a]Only miRNAs whose expression levels showed a greater than 3-fold increase or decrease in SW620 cells are listed.

(median 59 years) and none received preoperative chemotherapy. The studied group reflects the natural distribution of clinicopathological characteristics of CRC patients. Resected specimens were histologically examined by hematoxylin and eosin staining. Primary tumor tissues and corresponding nontumor mucosa were collected immediately after surgical removal and snap-frozen in liquid nitrogen until further use. Total RNA from the frozen tissues was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions.

Oligonucleotide construction and lentivirus production

miRNA mimics, the miRNA inhibitor and negative control miRNA oligonucleotides of has-miR-499-5p were from RiboBio Co. Ltd (Guangzhou, Guangdong, China). The small interfering RNAs (siRNAs) targeting FOXO4 and PDCD4 were synthesized by RiboBio Co. Ltd. An unrelated sequence was used as a negative control (provided by RiboBio). The sequences were FOXO4 siRNA, 5′-UCUCACCUCUUCCCAUUCCdTdT-3′ (sense); PDCD4 siRNA 5′-GGUGGCUGGAACAUGUAUUdTdT-3′ (sense); RNA duplex control, 5′-UUCUCCGAACGUGUCACGUdTdT-3′ (sense).

Stable transfectants overexpressing miR-499-5p were generated by lentiviral transduction using a pGCsil-GFP vector (GeneChem Co., Ltd, Shanghai, China). As a control, we also generated a lentiviral vector that expressed green fluorescent protein alone (LV-GFP). Transfection of oligonucleotides or lentivirus construction was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

In vitro migration and invasion assays

A 24 well transwell plate (8 mm pore size; Corning NY) was used to measure migratory and invasive ability of the cells. For transwell migration assays, 2.5×10^4 cells were added to the top chamber lined with a noncoated membrane. For invasion assays, chamber inserts were coated with 200 mg/ml of Matrigel (BD Biosciences, San Jose, CA), dried overnight under sterile conditions and 5×10^4 cells were placed in the top chamber. In both assays, cells were suspended in medium without serum or growth factors, and medium supplemented with serum was used as a chemoattractant in the lower chamber. After incubation at 37°C in 5% CO_2 for 24 h, the top chambers were swabbed to remove residual cells. Invading cells on the underside of the membrane were fixed in 100% methanol for 10 min, air-dried, stained with 0.1% crystal violet and counted with a microscope. Five random fields were analyzed for each insert. Assays were conducted in triplicate in three independent experiments.

Wound-healing assays

SW480–miR-499-5p or SW480–vector cells were seeded into 24 well culture plates and cultured until 90% confluent. A P200 pipette tip was used to make a vertical wound, and cells were washed several times with appropriate culture medium to remove cell debris. The extent of wound closure was monitored at designated times.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cells were grown in RPMI-1640 medium containing 10% fetal serum. For cell growth measurements, 1×10^3 cells were seeded in flat-bottom 96 well plates and incubated at 37°C in 5% CO_2 for 24 h; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide working solution was added to the medium, and the cells were incubated for 4 h. The medium was removed, and 150 µl
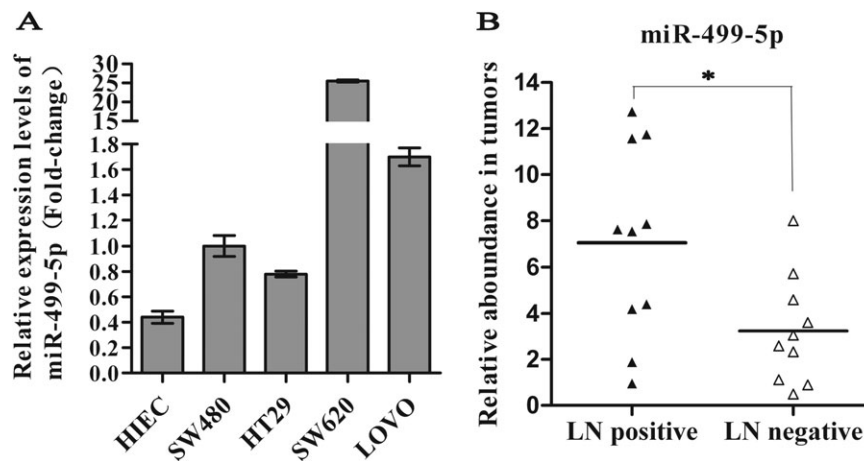
Fig. 1. MiR-499-5p expression in CRC cells and tissues. MiR-499-5p expression was investigated by qRT-PCR. (**A**) Each bar represents fold change relative to SW480 cell lines, calculated by $2^{-\Delta\Delta Ct}$. (**B**) Real-time qPCR analysis of miR-499–5p abundance in human CRC primary tumors, lymph node positive ($n = 10$) or negative ($n = 10$). Matching normal colorectal tissue sample from the same patient was used for normalization. Each sample was analyzed in triplicate and normalized to U6. Results were consistent with the microarray data. $^*P < 0.05$.

Table II. Relationship between clinicopathological parameters and miR-499-5p expression in primary CRC

Variable	Number of cases	%	Median expression of miR-499-5p (range) ($2^{-\Delta\Delta Ct}$)[a]	*P*-value
Age (years)				
≥60	45	50.0	4.76 (0.27–22.01)	0.257
<60	45	50.0	3.32 (0.26–38.05)	
Gender				
Male	50	55.6	4.70 (0.27–38.05)	0.346
Female	40	44.4	3.36 (0.26–16.68)	
Tumor size				
≥5	51	56.7	4.63 (0.26–38.05)	0.201
<5	39	43.3	3.32 (0.63–20.39)	
Degree of differentiation				
Well and moderately differentiated	54	60.0	3.50 (0.27–38.05)	0.394
Poorly differentiated	36	40.0	4.63 (0.26–20.82)	
TNM stage				
Stage I/II	44	48.9	2.28 (0.26–7.94)	<0.001
Stage III/IV	46	51.1	5.96 (0.64–38.05)	
Lymph node status				
No metastasis	46	51.1	2.38 (0.26–7.94)	<0.001
Metastasis	44	48.9	5.82 (0.64–38.05)	

TNM, tumor-node-metastasis. The data are shown as the median (range).

[a]$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{miR499-5p} - \Delta Ct\ calibrator)}$, $\Delta Ct_{miR499-5p} = (Ct_{miR499-5p} - Ct_{U6})$, $\Delta Ct\ calibrator = (Ct_{calibrator} - Ct_{U6})$.

dimethyl sulfoxide was added to dissolve the formazan crystals. Cell viability was measured by absorbance at 490 nm using a microplate reader (Model 680 Microplate Reader; Bio-Rad, Richmond, CA). The proliferation assay was repeated at least three times.

In vivo *tumor metastasis assays*

To produce experimental metastasis, SW480 cells transduced with lentiviral constructs carrying either miR-499-5p or vector control were harvested from tissue culture cells flasks and were washed and re-suspended in phosphate-buffered saline. Five-week-old BALB/C-nu/nu nude mice were purchased from the Shanghai Laboratory Animal Center of China and 2×10^6 cells suspended in 0.2 ml serum-free RPMI1640 were injected into the lateral tail vein of each mouse. Each tumor cell line was injected into 10 mice, and animals were maintained in a sterile animal facility. After 6 weeks, mice were killed, and lungs and liver were examined for metastases. Tumor tissues derived from various organs were dissected and examined histologically. Experiments were repeated two to three times.

Luciferase assay

Standard 3′-untranslated region (UTR) luciferase assays were performed to validate *in silico* target predictions. Plasmids containing wild-type Luc-FOXO4, mutant Luc-FOXO4, wild-type Luc-PDCD4 or mutant Luc-PDCD4 3′-UTR were specifically synthesized (GeneCopoeia) and used in luciferase reporter assays. These plasmids contain firefly luciferase and Renilla luciferase that functions as a tracking gene. Luciferase activity assays were performed following manufacturer's protocols. Briefly, SW480 cells were seeded in six-well plates, cotransfected with the lentiviral constructs containing miR-499-5p or vector contro and wild-type or mutated target gene 3′-UTR with Lipofect-amine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured 48 h after transfection using a Luc-Pair miR Luciferase Assay Kit (GeneCopoeia) according to the manufacturer's recommendations. Activities were normalized to Renilla luciferase. Results represent three independent experiments, each performed in triplicate.

Western blot

Cell proteins were extracted and separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and western blot analysis was performed according to standard procedures. Detection of β-actin on the same membrane was used as a loading control. Antibodies anti-FOXO4 (ab63254) or anti-PDCD4 (ab80590) were from Abcam (Cambridge, MA) and anti-β-actin (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analyses

Data are presented as mean ± standard error of the mean (standard error of the mean) or medians (range). Student's *t*-test (two tailed) or one-way analysis of variance test was employed to analyze *in vitro* and *in vivo* data unless otherwise indicated (χ^2 test). The nonparametric Mann–Whitney test was used to analyze the relationship between miRNA expression levels and various clinicopathologic characteristics. Statistical tests were performed with SPSS 12.0 software (Chicago, IL). $P < 0.05$ was defined as statistically significant. $*P < 0.05$; $**P < 0.01$.

Results

Microarray of miRNAs differentially expressed in CRC cell lines

To identify miRNAs potentially involved in CRC invasion and metastasis, we examined miRNA expression profiles of the CRC primary carcinoma cell line SW480 and the metastatic cell line SW620, using an miRNA microarray platform. Of the 875 human miRNAs on the array (Sanger miRBase release 13.0), a variety of miRNAs exhibited significantly different expression level between the two cell lines (Supplementary Table SII is available at *Carcinogenesis* Online). In particular, 9 miRNAs were upregulated and 12 miRNAs were downregulated more than 3-fold in SW620 cells versus SW480 cells (Table I). Of these, miR-499-5p displayed the most significantly different expression, with a log2 change of 8.05 in SW620 compared with SW480.

Microarray validation by real-time RT-PCR of miR-499-5p in CRC cell lines and tissues

To validate the miRNA microarray data, we performed qRT-PCR to analyze the expression levels of miR-499-5p in four human CRC cell lines (SW480, SW620, HT29, LOVO) and a normal HIEC. Cell lines were classified into three groups according to their metastatic propensities: (i) nontumorigenic HIEC; (ii) tumorigenic but minimally metastatic SW480 and HT29; and (iii) highly metastatic SW620 and LOVO cells. Basal expression levels of miR-499-5p were significantly increased in SW620 and LOVO cell lines compared with either low-metastatic (but tumorigenic) or immortalized (but nontumorigenic) cells (Figure 1A). To further confirm this observation, we used qPCR to investigate miR-499-5p expression in fresh tissues of primary tumors from 10 CRC patients with lymph node metastasis and 10 CRC patients without lymph node metastasis. The miR-499-5p level in each tumor sample was normalized to matching normal colorectal tissue sample from the same patient. We found that levels of miR-499-5p expression were significantly higher in CRC with lymph node metastasis than in CRC without lymph node metastasis (Figure 1B) ($P = 0.021$). These results suggested that miR-499-5p may play a causal role in CRC metastasis and invasion.

MiR-499-5p expression in CRC associated with clinical stage and lymph node metastasis

To determine the potential clinicopathological implications of altered miR-499-5p expression, we investigated the expression of miR-499-5p in an additional 70 CRC tissues by qRT-PCR. These were quantitated by the $2^{-\Delta\Delta Ct}$ term, together with the previous 20 samples. One
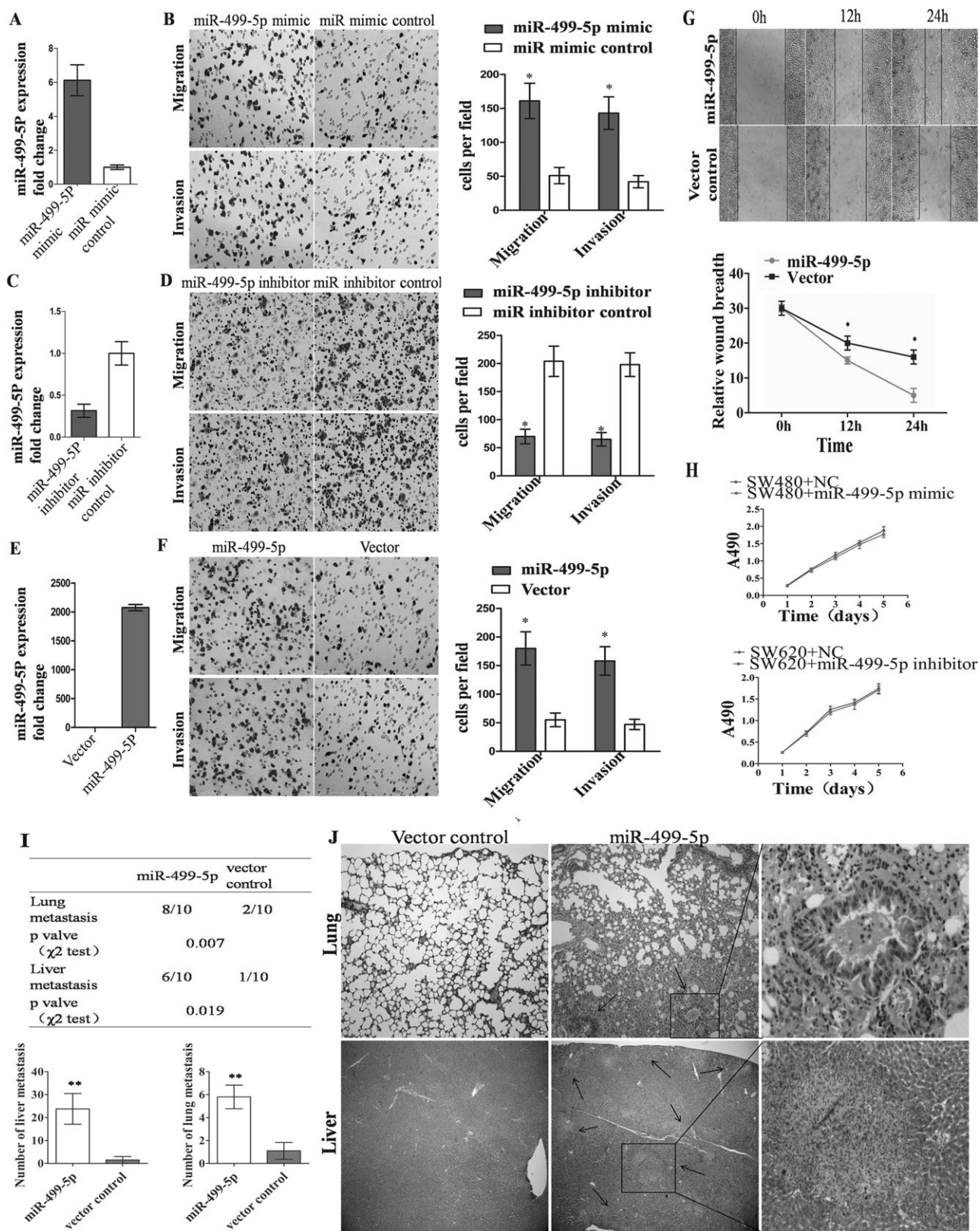
Fig. 2. miR-499-5p promoted CRC cells invasion and metastasis. (**A**) qRT-PCR analysis of miR-499-5p in SW480 cells transfected with a miR-499-5p mimic or a negative control. (**B**) Invasion and migration assay of SW480 cells transfected with a miR-499-5p mimic or a negative control. Representative fields of migration (top) or invasive (bottom) cells on membrane (left). Average number of invasive or migration cells number per field from three independent experiments ± standard error is at right. $^*P < 0.05$. (**C**) qRT-PCR analysis of miR-499-5p in SW620 cells transfected with miR-499-5p inhibitor or a negative control. (**D**) Invasion or migration assay of SW620 cells transfected with miR-499-5p inhibitor or a negative control. $^*P < 0.05$. (**E**) QRT-PCR analysis of miR-499-5p in SW480 cells transfected with the lenti-miR-499-5p-expression vector or the miRNA control vector. (**F**) Invasion or migration assay of SW480 cells transfected with the lenti-miR-499-5p-expression vector or the miRNA control vector. $^*P < 0.05$. (**G**) Wound-healing assay to evaluate the effect of miR-499-5p expression on cell migration capacity. Cell culture plates with SW480–miR-499-5p cells or SW480-vector cells were wounded and incubated. Healing was determined at the
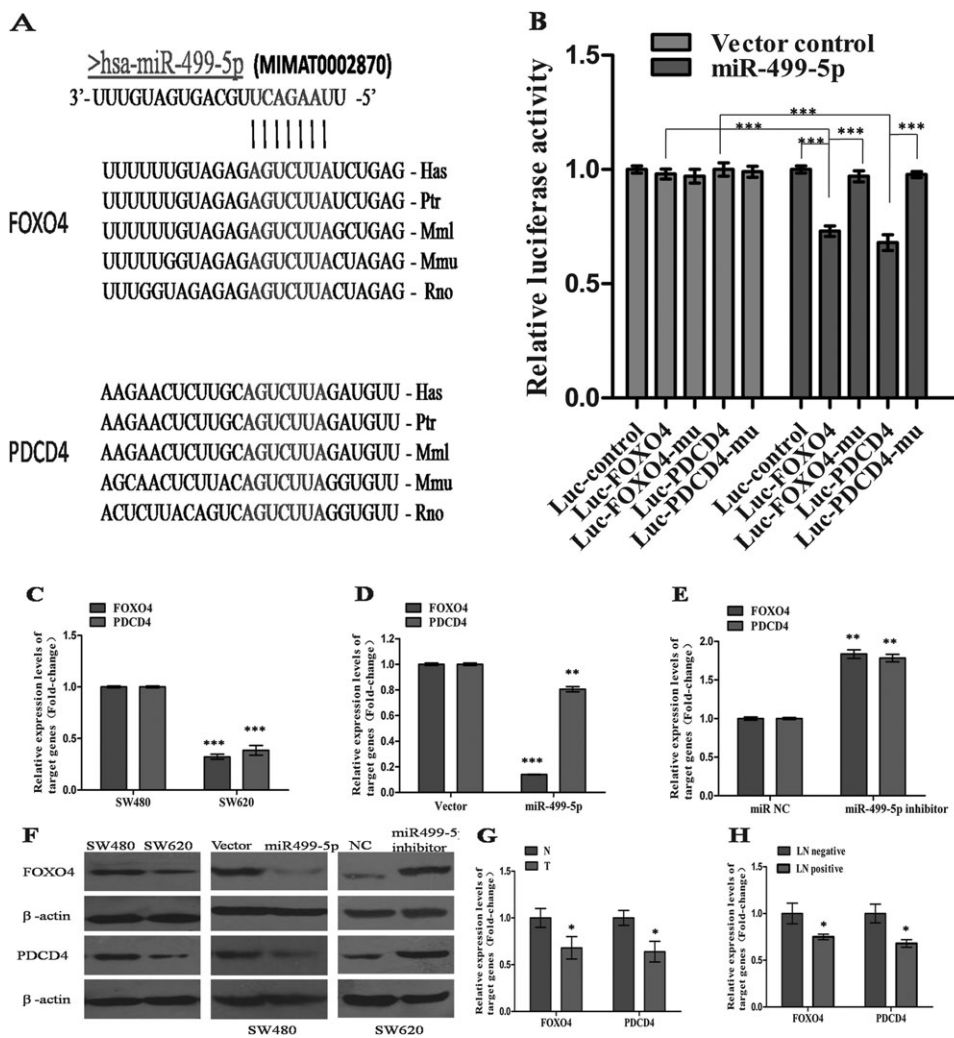
Fig. 3. FOXO4 and PDCD4 are targets of miR-499-5p. (**A**) MiR-499-5p target site resides at nucleotides 383–389 of the FOXO4 3'-UTR and 17–23 of the PDCD4 3'-UTR are highly conserved in different species. Upper panel, sequence alignment of miR-499-5p with binding sites on the FOXO4 3'-UTR of five species. Lower panel, sequence of the miR-499-5p binding site in the PDCD4 3'-UTR of five species. Hsa, human; Ptr, *Pan troglodytes*; Mml, *Macaca mulatta*; Mmu, *Mus musculus*; Rno, rat. (**B**) Luciferase activity assay for direct targeting of the 3'-UTR of FOXO4 and PDCD4 by miR-499-5p. Two copies of wild-type and mutant miR-499-5p target sequences of FOXO4 or PDCD4 were fused with luciferase reporter and transfected into vector control and miR-499-5p stably infected SW480 cells, and luciferase activity measured (***$P < 0.001$, t-test). (**C–E**) FOXO4 and PDCD4 mRNA levels analyzed by qRT–PCR; (**F**) FOXO4 and PDCD4 protein levels analyzed by western blot. FOXO4 and PDCD4 expression was lower in SW620 cells compared with SW480 cells and decreased with the lenti-miR-499-5-expression vector. The reverse was observed for FOXO4 and PDCD4 expression in miR-499-5p knockdown. (**G** and **H**) Expression of FOXO4 and PDCD4 in CRC specimens. (**G**) RT-PCR of relative expression of FOXO4 and PDCD4 in 10 CRC tissues (T) compared with adjacent noncancerous tissues (N). (**H**) Relative expression of FOXO4 and PDCD4 in lymph node (LN)-positive CRC tissues compared with lymph node negative tissues. Each sample was analyzed in triplicate and normalized to glyceraldehyde 3-phosphate dehydrogenase.

of the CRC tissues was chosen as the calibrator and was given a relative expression value of 1. The association of miR-499-5p with clinicopathologic factors (Table II) was examined in tumor tissues, and miR-499-5p overexpression was associated with advanced clinical tumor-node-metastasis stage and lymph node metastasis ($P < 0.001$ for both by Mann–Whitney test). MiR-499-5p expression in CRC patients did not correlate with age, gender, tumor size or cell differentiation.

miR-499-5p promoted CRC migration and invasion in vitro
To determine whether miR-499-5p changed the capacity of CRC cells for migration and invasion, miR-499-5p mimics were transiently transfected into SW480 cells, and qRT-PCR was used to demonstrate

miRNA overexpression (Figure 2A). Overexpression of miR-499-5p substantially increased the migratory ability of CRC cells as indicated by an increase in migrated cells in a transwell cell migration assay. A similar result was also was observed in a cell invasion assay (Figure 2B). We transiently transfected miR-499-5p inhibitors into the SW620 line, which has relatively higher endogenous miR-499-5p expression. Successful inhibition of endogenous miR-499-5p expression was confirmed by qRT-PCR (Figure 2C). Inhibition of miR-499-5p significantly impeded cell migration and invasion in SW620 cells (Figure 2D). We prepared stable miR-499-5p-expressing transfectants using a lentivirus system and established a stable SW480 cell line that overexpressed miR-499-5p (SW480–miR-499-5p), as seen by qRT-PCR (Figure 2E).

←

indicated times. (**H**) Proliferation rates of cell sublines detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (**I**) Incidence of metastasis in mice (upper panel) and mean number of visible tumor nodules in liver and lung (lower panel). ** $P < 0.01$. Student's t-test ($n = 10$ per group). SW480 cells were transfected with the lenti-miR-499-5p expression vector or the miRNA control vector and injected into nude mice via the tail vein for an *in vivo* metastasis assay. Animals were killed 6 weeks after injection. (**J**) Representative hematoxylin and eosin staining of lungs and livers isolated from mice injected with SW480–vector or SW480–miR-499-5p cells. Arrows indicate tumor foci in lungs and livers. Magnification: ×100 (left), ×200 (right).
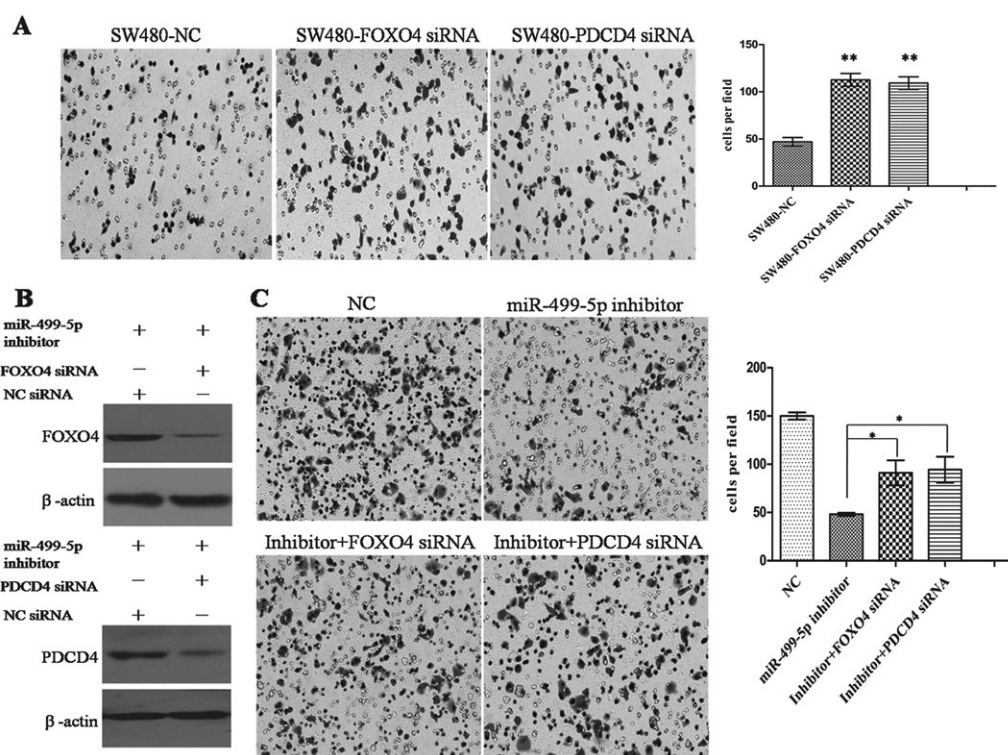
Fig. 4. Prometastasis effect of miR-499-5p mediated by inhibiting target genes FOXO4 and PDCD4. (**A**) Migration assay. Knockdown of FOXO4 and PDCD4 by siRNA in SW480 cells significantly promoted cell migration. Representative fields of migration cells on the membrane (left). Average number of migration cells number per field from three independent experiments ± standard error (right).**P < 0.01. (**B**) SW620 cotransfected with miR-499-5p inhibitor and FOXO4, PDCD4 siRNA or a negative control (NC) siRNA. Western blot analysis for FOXO4 and PDCD4 showing levels of FOXO4 and PDCD4. (**C**) Cell migration assay with FOXO4 and PDCD4 siRNA reversing miR-499-5p knockdown-mediated suppression of cell migration. *P < 0.05.

SW480–miR-499-5p consistently demonstrated higher cell migration and invasion than a SW480 line stably transfected with empty vector (Figure 2F).

Additionally, wound-healing assays showed that SW480–miR-499-5p closed wounds more rapidly than control cells (Figure 2G), indicating that overexpression of miR-499-5p increased the ability of SW480 cells to migrate into the wound. We found that expression of miR-499-5p in SW480 cells using a miR-499-5p mimic did not induce tumor cell growth, and knockdown of miR-499-5p expression in SW620 cells with a miR-499-5p inhibitor did not significantly reduce cell viability (Figure 2H). These results indicated that miR-499-5p significantly enhanced CRC cell migration and invasion *in vitro*.

miR-499-5p promoted CRC migration and invasion in a nude mouse xenograft model

To further explore the role of miR-499-5p in tumor invasion and metastasis *in vivo*, we injected SW480–miR-499-5p cells that were stably expressing miR-499-5p or vector-transfected control cells into nude mice through the lateral tail vein. The number and size of lung and liver metastatic nodules dramatically increased in the SW480–miR-499-5p group compared with the vector controls (Figure 2 I and J). Taken together, these observations suggested that miR-499-5p is a positive metastatic regulator for CRC.

FOXO4 and PDCD4 were targets of miR-499-5p in CRC metastasis

The preceding findings indicated that miR-499-5p acted as a metastasis promotor in CRC. Therefore, we searched for potential gene targets of miR-499-5p that might contribute to its prometastatic function. We performed *in silico* studies to search for potential gene targets of miR-499-5p using the bioinformatics algorithms TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/

home.do). Both algorithms identified FOXO4 and PDCD4 as targeted by miR-499-5p, based on putative target sequences at 383–389 bp of the FOXO4 3′-UTR and 17–23, 467–473 and 533–539 bp of the PDCD4 3′-UTR. The sequences were evolutionarily conserved in different species, as shown by TargetScan 5.1 (Figure 3A).

FOXO4 is considered to be a tumor-suppressor protein (20–22)and is implicated in the metastasis of cholangiocarcinoma (23). PDCD4 is a well-known tumor suppressor that can suppress tumor growth (24–26), invasion and metastasis (27,28). These findings suggest that the prometastatic function of miR-499-5p in CRC may be attributed to a suppressive effect on FOXO4 and PDCD4 expression. To test this hypothesis, luciferase reporter assays were performed on SW480–miR-499-5p stable transfectants with luciferase reporters carrying the FOXO4 or PDCD4 3′-UTR. Both reporters had significantly decreased activity compared with an SW480 strain stably transfected with an empty vector control. This suppressive effect was significantly reversed in an miR-499-5p target mutant (Figure 3B).

Additionally, we analyzed the endogenous expression of FOXO4 and PDCD4 in SW480 and SW620 CRC cells. The results showed a negative correlation between miR-499-5p levels and FOXO4 and PDCD4 mRNA in these cells (Figure 3C). Furthermore, cells stably expressing miR-499-5p (SW480–miR-499-5p) showed a clear attenuation of FOXO4 and PDCD4 mRNA (Figure 3D) and protein expression (Figure 3F), while transient inhibition of miR-499-5p in SW620 caused a moderate increase in FOXO4 and PDCD4 mRNA (Figure 3E) and protein expression (Figure 3F). This finding supported the hypothesis that FOXO4 and PDCD4 expression were inversely associated with miR-499-5p expression in CRC cells.

To determine the clinical significance of miR-499-5p target genes, we examined FOXO4 mRNA levels in 10 pairs of matched CRC specimens using RT-PCR. The level of FOXO4 mRNA was significantly downregulated in CRC tissues compared with adjacent

noncancerous tissues (Figure 3G). CRC specimens were subsequently divided into lymph node-positive ($n = 5$) or negative ($n = 5$) groups based on patient lymph node status. The level of FOXO4 mRNA was dramatically downregulated in lymph node-positive CRC tissues compared with lymph node-negative tissues. Similar to FOXO4, we also detected downregulation of PDCD4 in lymph node-positive CRC tissues (Figure 3H).

To explore the biological functions of FOXO4 and PDCD4 in CRC cells, we determined whether inhibition of FOXO4 and PDCD4, similar to miR-499-5p expression, promoted CRC cell migration. In SW480 cells, *in vitro* knockdown of FOXO4 and PDCD4 promoted cell migration (Figure 4A). To further test this, we cotransfected SW620 cells with siRNA for FOXO4 mRNA and miR-499-5p inhibitor and found that the effect of miR-499-5p inhibitor was partially attenuated by siRNA for FOXO4 mRNA (Figure 4B and C). Similar results were observed in cells cotransfected with siRNA for PDCD4 mRNA and miR-499-5p inhibitor. These data confirmed that the prometastasis effect of miR-499-5p was mediated by inhibiting target genes FOXO4 and PDCD4.

Discussion

The major causes of death from cancer are complications arising from metastasis (29). Recently, miRNAs have been found to promote (15,30) or suppress (13,31–33) tumor metastasis, providing a new perspective on the metastatic process. The cell lines SW480 and SW620 differ in their metastatic capability, so the genes that differ between them should correlate with metastasis. We investigated the miRNA expression profiles of SW480 and SW620 using an miRNA microarray and identified miR-499-5p as having the most dramatic upregulation in SW620.

qRT-PCR was used to detect the precise expression of miR-499-5p in colon cancer cell lines SW480, SW620, HT29 and LOVO and in the normal human intestinal epithelial cell line HIEC. MiR-499-5p levels varied significantly among the different cell lines and correlated with metastatic ability. In the high invasion-potential cell lines SW620 and LOVO, miR-499-5p expression was dramatically higher than in low invasion-potential cell lines SW480 and HT29, and HIEC cells had the lowest expression level. This suggested a causal role for miR-499-5p in human CRC cell migration and invasion in culture.

We determined miR-499-5p levels in 90 primary human CRC tissues by qRT-PCR. The relationship between miR-499-5p expression and clinicopathologic factors was examined in CRC patients. While there was no relationship between miR-499-5p expression and gender, tumor size or differentiation grade, we found a significant association between miR-499-5p expression and tumor-node-metastasis stage and lymphatic metastasis.

Whether upregulation or downregulation of miR-499-5p also affects colon cancer migration and invasion is unclear; therefore, we used miR-499-5p mimics, lenti-miR-499-5p and inhibitor constructs to perform miR-499-5p gain-of-function or loss-of-function studies in human CRC cells. Our findings showed that expression of miR-499-5p increased migration and invasion capacity of SW480 cells *in vitro* and *in vivo*, whereas knockdown of miR-499-5p expression in SW620 reduced migration and invasion. In contrast, miR-499-5p showed limited ability to affect CRC cell growth. Thus, all lines of evidence in this study suggested that miR-499-5p functions primarily as a prometastatic miRNA in CRC.

Having shown a crucial function for miR-499-5p in promoting CRC metastasis, we sought possible gene effectors participating in this function. A single miRNA can regulate a multitude of target genes concomitantly; for instance, miR-31 can suppress breast cancer cell motility by targeting RhoA, ITGA5, Fzd3 and RDX simultaneously (34). Our bioinformatic analyses indicated FOXO4 and PDCD4 as potential targets of miR-499-5p.

FOXO4 is a member of the Forkhead box O-class (FOXO) subfamily that also includes FOXO1, FOXO3 and FOXO6. FOXO proteins function as tumor suppressors by inhibiting cell proliferation, promoting apoptosis and protecting cells from DNA damage and oxidative stress (20–22). Many studies suggest that the FOXO factors may play critical roles downstream of the PI3K-Akt pathway (35,36), and Akt inhibits the tumor suppressor function of the FOXO factors via phosphorylation, which leads to nuclear exportation of FOXO, together with the chaperone protein 14-3-3(37–40).

Lee *et al.* (23)found that ANXA8 is transcriptionally downregulated by epidermal growth factor-mediated FOXO4 phosphorylation that leads to the deregulation of FAK and F-actin dynamics, which is responsible for epithelial-to-mesenchymal transition morphology, suggest that FOXO4 and ANXA8 play key roles in growth factor-mediated tumor metastasis during the epithelial-to-mesenchymal transition change in CRC.

PDCD4 was first identified as a tumor suppressor gene in a mouse keratinocyte (JB6 cells) model, in which high PDCD4 levels rendered cells resistant to transformation by the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (41). PDCD4 expression levels are reduced in cell lines derived from many different tumor types (24–26). Studies in cultured ovarian cancer cells, for example, suggest that PDCD4 suppresses proliferation and cell cycle progression and induces apoptosis (42). PDCD4 levels are also decreased in tumor samples from colon cancer (43,44), esophageal carcinoma (30), pancreatic cancer (45), hepatocellular carcinoma (26), lung cancer (24), breast carcinoma (46) and glioma (47).

Besides its well-characterized functions as a suppressor of cell transformation, and as an inducer of apoptotic cell death, increasing evidence suggests PDCD4 is also a suppressor of migration and invasion, the two decisive initial steps of the metastatic cascade. Yang *et al.* (27) showed that in colon cancer cells, *in vitro* invasion into Matrigel is suppressed by PDCD4, at least in part through downregulation of MAP4K1, MMP2 and potentially other matrix metalloproteinases. Wang *et al.* (44) showed that downregulation of PDCD4 leads to an increase in invasion by HT29 colon cancer cells, which involves the regulation of E-cadherin, β-catenin and AP-1-dependent transcription. In hepatocellular carcinoma cells, PDCD4 has recently been shown to correlate inversely with metastatic capacity (28). Allgayer *et al.* (48) showed that the inhibition of invasion and intravasation by PDCD4 is due at least in part to regulation of the urokinase receptor (u-PAR), one of the major invasion-related genes in many solid cancers. U-PAR binds the specific ligand u-PA which, when bound by the receptor, converts plasminogen to active plasmin and initiates a cascade of proteolytic activities that lead to the highly efficient degradation of extracellular matrix components.

In this report, we demonstrated that miR-499-5p binds to the 3′-UTR of FOXO4 and PDCD4, dramatically decreasing the level of FOXO4 and PDCD4 mRNA and protein expression. This provides the first line of evidence in support of a mechanism for FOXO4 and PDCD4 regulation at the posttranscriptional level.

We also found that knockdown of FOXO4 or PDCD4 gene expression by siRNA reversed miR-499-5p-inhibitor-mediated suppression of tumor cell migration. In summary, we identified an important prometastatic miRNA, miR-499-5p, whose expression was frequently increased in CRC. MiR-499-5p overexpression promoted cell migration and invasion in human CRC cell lines. Additionally, we identified FOXO4 and PDCD4 mRNA as direct and functional targets of miR-499-5p. Although our understanding of the complicated mechanisms underlying CRC progression are still limited, our data may provide a strategy for targeting miR-499-5p as a novel therapeutic application to treat advanced CRC patients.

Supplementary material

Supplementary Tables SI and SII can be found at http://carcin.oxford-journals.org/

Funding

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