Original Article

PDZRN4-mediated colon cancer cell proliferation and dissemination is regulated by miR-221-3p

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Background: Suppression of *PDZRN4* expression in colon cancer tissues may be associated with elevated levels of the microRNA 221 (miR-211). To uncover potential targets for treatment, the present study investigated *PDZRN4* in colon cancer development, and explored the role of miR-221-3p in the regulation of *PDZRN4*.

Methods: RNA expression arrays were searched in the NCBI database, and *PDZRN4* (PDZ domain containing ring finger 4) was selected as a potential downregulated gene in colon cancer. *PDZRN4* mRNA and protein in colon cancer and matched normal tissues were analyzed. The proliferation and dissemination of HCT116 cells overexpressing *PDZRN4* was assessed via functional assays. Bioinformatics analysis and luciferase reporter assay were applied to determine the regulatory link between miR-221-3p and *PDZRN4* mRNA.

Results: There was significantly less *PDZRN4* mRNA and *PDZRN4* protein in colon cancer tissue compared with normal tissues. HCT116 cells overexpressing *PDZRN4* were less able to disseminate relative to the control. Expression of *PDZRN4* was directly inhibited by miR-221-3p. Knockout of miR-211-3p was associated with attenuated proliferation and dissemination of HCT116 cells.

Conclusions: *PDZRN4* may function as a tumor suppressor and is downregulated in colon cancer tissues, possibly due to dysregulation via miR-221-3p. This study provides new insight into colon cancer development.

Keywords: PDZ domain containing ring finger 4 (*PDZRN4*); colon cancer; microRNA; 3'-untranslated region (3'-UTR); mRNA; proliferation

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Introduction

Despite rapid advances in diagnostic and treatment strategies, colon cancer still ranks high among commonly diagnosed cancers, and its incidence is rising (1). In China, the incidence in men and women is 16.9 and 11.6 per 100,000, respectively (2). Colon cancer is closely associated with other diseases such as diabetes and obesity, making it a major public health risk (3,4). Despite the rapid increase in newly diagnosed patients, the etiology and mechanism of colon cancer development remains unclear.

The gene *PDZRN4* (PDZ domain containing ring finger 4) belongs to the ligand of numb protein-X (LNX) family and is dysregulated in multiple diseases, including radiation-induced papillary thyroid carcinoma, rectal adenocarcinoma, and hepatocellular carcinoma (5,6). In human liver cancer cell lines, the ectopic expression of *PDZRN4* inhibited cancer cell proliferation, as well as plate colony formation and anchorage-independent colony

formation (7). Analysis of gene expression data indicates that the gene *PDZRN4* is downregulated in colon cancer. In the present study, we investigated the role of *PDZRN4* in colon cancer development.

MicroRNAs (miRNAs) are a small noncoding RNA that, by binding to a coding sequence or 3'-untranslated region (UTR), can interfere with mRNA translation or stability, changing expression of the gene. Depending on its downstream gene, miRNAs can function as either tumor suppressors or oncogenes. Because miRNAs have such roles in the development of cancers (8-10), we wondered whether miRNAs may be implicated in the differential expression of *PDZRN4* in colon cancer.

Specifically, miR-221 is a putative regulator in tumor development (11-13), and according to open access data in the National Center for Biotechnology Information (NCBI) database its levels are usually elevated in colon cancer (GSE101502). In addition, higher level of miR-221 in colon cancer tissues is associated with poor prognosis (14,15). The expression of miR-221 is regulated by multiple regulators, including KRAS, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), STAT3 (signal transducer and activator of transcription 3), and KRAP (Ki-ras-induced actin-interacting protein), while KRAP-regulated miR-221 expression could only be seen in a 3D culture, and NF-κB and STAT3 activation is, in turn, manipulated by miR-221 expression (16,17).

It is possible that miRNAs can have multiple targets, and the link between miR-221 and *PDZRN4* remains unclear. To contribute information toward the diagnosis and treatment of colon cancer, the present study explored the potential regulation of *PDZRN4* by miR-221-3p.

Methods

Ethics statement

The Institutional Ethics Committee of Second Affiliated Hospital of Soochow University (Suzhou, 215004, Jiangsu Province, China) reviewed and authorized the clinical section of this research.

Colon cancer tissue samples

Twenty pairs of colon cancer tissues and adjacent normal tissues were collected from patients who received treatment in our hospital. None of the patients received radiotherapy or chemotherapy before surgery (*Table S1*). All diagnoses were obtained via pathological examination. All examinations and evaluations of clinical samples were conducted using paired normal tissue as a control.

Cell line

Cells of the human colon cancer cell line, HCT116 were purchased from the American Type Culture Collection and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37 ℃, 5% CO_2.

293T cells were previously preserved in our lab and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% FBS and 2 mM L-glutamine at 37 ℃, 5% CO_2.

Cells were observed with a microscope (Olympus, Tokyo, Japan) and green fluorescent protein (GFP) was observed with a fluorescence microscope.

Plasmids

The pLVX-*PDZRN4* plasmid was constructed by inserting the *PDZRN4* coding sequence into the lentiviral expression vector, pLVX-IRES-ZsGreen1. The pLVX-miR-221-3p sponge was constructed by referring to a published article (18).

Immunohistochemistry (IHC)

The cancer tissues and paired adjacent normal tissues obtained from patients during surgery were processed for staining with hematoxylin and eosin and IHC. All the samples were formalin-fixed, paraffin-embedded, and immunostained with PDZRN4 antibody (ThermoFisher Scientific, Waltham, MA, USA). Quantification was conducted by counting the positive cells per field. Data is shown as mean ± standard deviation (SD).

Western blot

Cells were lysed with sodium dodecyl sulfate (SDS) lysis buffer (Beyotime, China) and denaturalized in a water bath at 100 ℃. The prepared protein was resolved via SDS-PAGE, transferred to a polyvinylidene difluoride membrane from Millipore, and incubated with PDZRN4 primary antibody and HRP-Goat Anti-Rabbit IgG (H + L) (MultiSciences, Hangzhou, China). Protein levels were detected with a peroxide LumiGLO reagent (Cell Signaling

Table 1 Primers used for RT-qPCR

Primers	Sequences
PDZRN4-forward	TTTGCCCTGGAGCGCTTCGCAGA
PDZRN4-reverse	CGCCTCCAGCTCGTGCAGCCTGA
miR-221-3p-forward	CAAGGAATCATGTATGCTGTAG
miR-221-3p-reverse	AGGATGACATTACACCTTATCTC
U6-forward	GCTTCGGCAGCACATATACTAAAAT
U6-reverse	CGCTTCACGAATTTGCGTGTCAT

Technology, 7003). Quantification of the relative protein levels was conducted according to the ratio of gray-value to the corresponding internal references. A representative graph was constructed and protein was quantified based on five pairs of clinical samples or three independent experiments and shown as mean ± SD.

Reverse transcription followed by real-time quantitative PCR (RT-qPCR)

RNA was extracted in accordance with the instructions for TRIzol reagent use (GENEray, Shanghai, China). The total RNA was subjected to reverse transcription using a kit from GENEray, Shanghai, China. Finally, cDNA was used for qPCR analysis. The RNA levels were normalized to internal references, and shown as mean ± SD (for primers, *Table 1*).

Fluorescence-activated cell sorting (FACS, flow cytometry)

The rate of GFP-positive cells was determined using Accμri C6 (BD, San Jose, CA, USA). Cells were digested into single cells and determined by the GFP channel of Accμri C6. GFP-positive cells and the total cell number were counted automatically. The percentage of positive cells was calculated as positive cells number/total cell number ×100%.

Cell proliferation assay using cell-counting kit 8 (CCK8)

Two thousand cells were suspended in 100-μL culture medium and seeded per well in a 96-well plate; 8 plates were seeded. Each plate was collected daily after seeding, 10 μL CCK8 solution (MultiSciences, Hangzhou, China) was added, and measurements were performed using a microplate reader at 450 nm; the first measurement was done a few hours after seeding. The relative proliferation rate is shown as mean ± SD of reads at 450 nm.

Plate clone formation assay

One hundred cells were suspended in 2 mL culture medium in a 6-well plate. The medium was replenished every 2 days. The cell culture was observed daily until visible clones had formed. The culture medium was carefully removed and clones were stained with crystal violet solution. Data is shown as mean ± SD of numbers of clones per field.

Migration and invasion assay

Cells were seeded to the upper chamber of Millicell hanging cell culture inserts (Merck Millipore, Darmstadt, Germany). Culture medium without FBS and complete culture medium, were added, respectively, to the upper and lower chambers of the culture wells. Cells that had migrated to the lower surface of the filter were fixed with 70% methanol and then stained with 0.5% crystal violet solution.

For the invasion assay, hanging cell culture inserts were pre-coated with diluted Matrigel an hour before cell seeding. The cells that migrated or invaded the lower surface of the wells were counted. Data are shown as mean ± SD. Student's *t*-test was performed when comparing data between groups.

Wound-healing assay

Cells were seeded to six-well culture plates and cultured overnight. By the second day, the culture medium was carefully removed and medium pipette tips were used to scratch the monolayer cell culture. Floating cells were carefully removed and fresh medium was added to the cell culture.

Graphs were constructed at the time of the scratch (0 hour) and 12 hours after treatment.

The percentage of scratch remaining after wounding was calculated as: (measurement at 12 hours/measurement at 0 hour) ×100%. The percentage of wound closure was calculated as: 100% – percentage of wound remaining. The percentage of wound closure is shown as mean ± SD.

Statistical analysis

The data in this study are shown as mean ± SD. The comparison of means between two groups was conducted using Student's *t*-test. $P<0.05$ was considered significant; *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

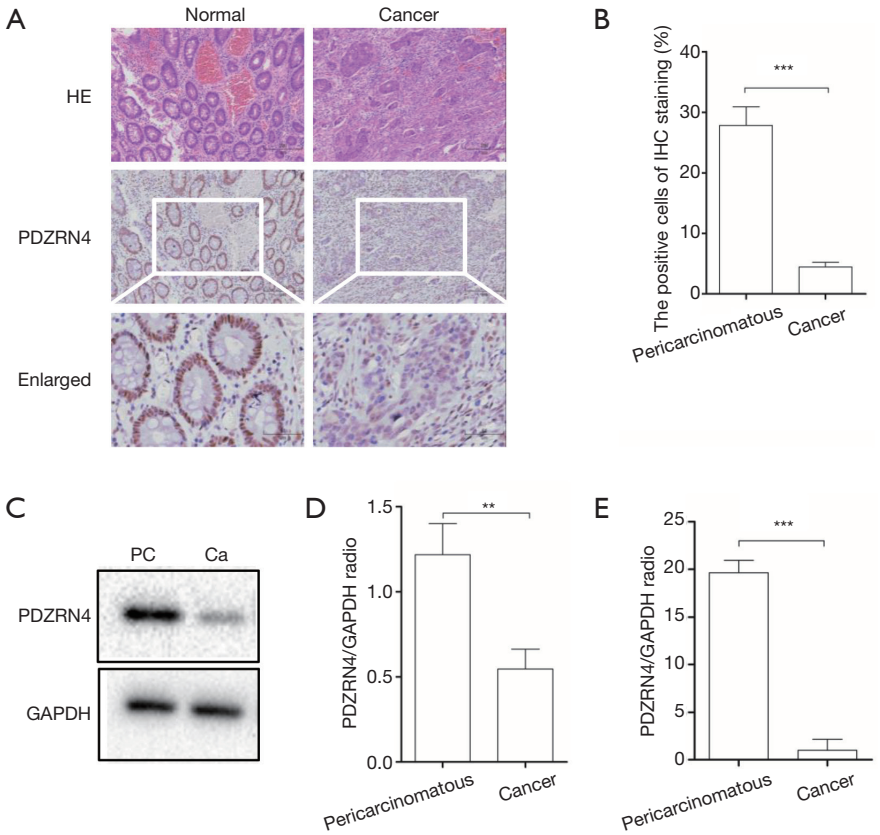


Figure 1 PDZRN4 expression is inhibited in colon cancer tissues. (A) PDZRN4 protein in normal colon tissue (left) and colon cancer tissue (right) revealed by IHC (×100 and ×400 for enlarged). (B) Quantification of cells (as in *Figure 1A*) stained positive for PDZRN4 using normal tissue as the control. ***P<0.001 for Student's *t*-test. (C) Western blot of PDZRN4 protein in five pairs of normal and cancer colon tissues. Ca, five colon cancer tissues; PC, matched normal tissues. (D) Quantification of PDZRN4 protein (see *Figure 1C*) using normal tissue as the control. **P<0.01 for Student's *t*-test. (E) mRNA levels of PDZRN4 in the same five pairs of normal and cancer colon tissues by RT-qPCR (see *Figure 1C*). ***P<0.001 for Student's *t*-test.

Results

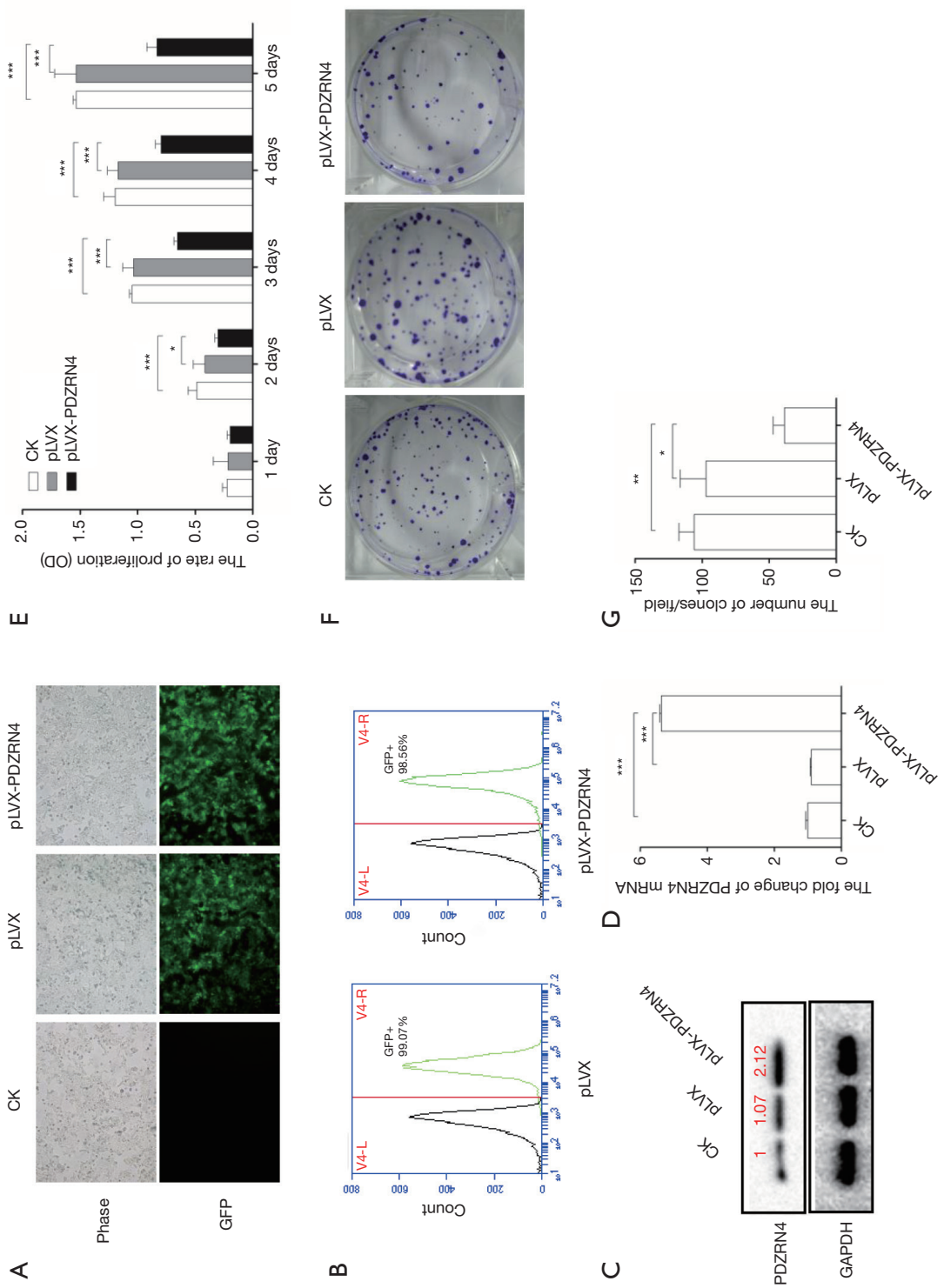
PDZRN4 is downregulated in colon cancer at both the mRNA and protein level

In the initial phase of this investigation, mRNA array data obtained from NCBI was analyzed (GEO accession: GSE75970 and GSE74604). We compared gene expression levels in both the array and screened differentially expressed gene, genes having a 2-fold difference in quantity between colon cancer tissues and normal tissues (*Figures S1*,*S2*). After searching studies and reports concerning differentially expressed genes, we found PDZRN4, downregulated in both arrays, had never been studied in colon cancer, and was not well illustrated in almost any disease.

The levels of PDZRN4 protein in 5 pairs of colon cancer tissues and matched normal tissues were compared using IHC and western blot (*Figure 1A*,*B*,*C*,*D*). Both analyses showed lower amounts of PDZRN4 protein in the colon cancer tissues than in the normal tissues. In addition, RT-qPCR of 20 pairs of samples showed less *PDZRN4* mRNA in the cancer samples (*Figure 1E*). These results suggest that *PDZRN4* is suppressed at both the mRNA and translated levels in colon cancer.

Ectopic expression of PDZRN4 attenuated HCT116 cell's tumorigenesis

To determine if *PDZRN4* may be a regulator in colon cancer development, the gene was overexpressed in HCT116 cells (*Figure 2A*). The FACS, western blot, and RT-qPCR results confirmed the successful overexpression of *PDZRN4* (*Figure 2B*,*C*,*D*, respectively).



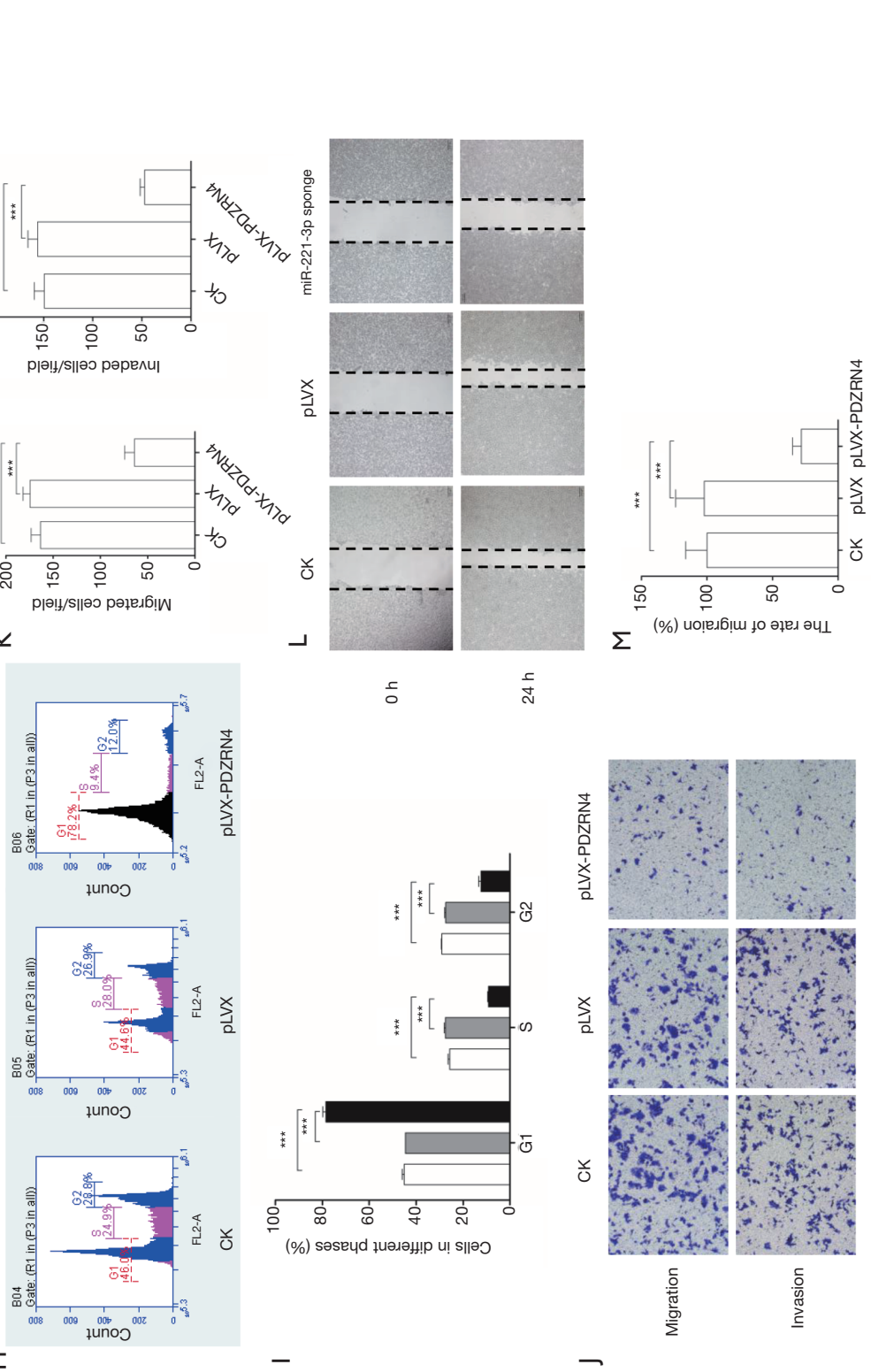


Figure 2 Overexpression of PDZRN4 inhibited the proliferation, migration, and invasion of HCT116 cells. (A) HCT116 cells were transduced with lentiviruses (middle) pLVX empty vector and (right) pLVX-PDZRN4. Representative images of (left) untreated cells, pLVX empty vector, or pLVX-PDZRN4 transduced cells were taken under (Phase, top) light microscope and (GFP, bottom) fluorescent microscope (100×). (B) Percentages of cells treated as in *Figure 2A* tested positive for GFP using FACS. (C) Western blot of PDZRN4 proteins in cells treated as in *Figure 2A*. (D) mRNA levels of PDZRN4 in cells treated as in *Figure 2A*. ***P<0.001 for Student's *t*-test. (E) Proliferation rates of cells treated as in *Figure 2A* by cell proliferation assay. *P<0.05, ***P<0.001 for Student's *t*-test. (F) Representative images for the clone formation assay of cells treated as in *Figure 2A*. (G) Numbers of clones formed (*Figure 2F*). *P<0.05, **P<0.01 for Student's *t*-test. (H) Percentage of cells treated as in *Figure 2A* in different cell cycle phases. (I) Percentages of cells in different cell phases. ***P<0.001 for Student's *t*-test. (J) Representative images for transwell migration and invasion of cells treated as in *Figure 2A* (100×). (K) Number of migrated and invaded cells per field. ***P<0.001 for Student's *t*-test. (L) Representative images of wound healing assay for cells treated as in *Figure 2A* (×100) ***P<0.001 for Student's *t*-test.

To investigate how *PDZRN4* regulates cancer cell activities, the cells overexpressing *PDZRN4* were then applied for functional assays. The cells overexpressing *PDZRN4* proliferated less compared with the control cells, as measured by the cell proliferation (*Figure 2E*) and plate clone formation (*Figure 2F,G*) assays. A study of the cell cycle of the transformed cells showed that fewer of those overexpressing *PDZRN4* reached S phase, compared with the control. This suggests that there was less DNA replication in the overexpressing cells (*Figure 2H,I*).

Tumor cells often have an enhanced ability to disseminate. In the present study, cells with overexpressed *PDZRN4* were much less able to disseminate via migration and invasion (*Figure 2J,K*), as also indicated by the wound healing assay (*Figure 2L,M*). Altogether, these data suggest that *PDZRN4* is suppressed in colon cancer, and this suppression promotes cancer cell proliferation and dissemination. Thus, *PDZRN4* may function as a tumor suppressor.

With this supposition, the regulation of *PDZRN4* expression in colon cancer was investigated.

PDZRN4 mRNA is a direct target of miR-221-3p

Since *PDZRN4* expression was dysregulated at both the mRNA and protein levels, we investigated whether it may be regulated by miRNAs. Thus, data from the noncoding RNA expression profile (GEO accession: GSE101501) was explored for implications in colon cancer. Using the databases RNAhybrid, TargetScanHuman, miRWalk, and miR, the following miRNAs listed as upregulated in colon cancer tissues in the databases were selected and investigated for binding potential with the *PDZRN4* 3'-UTR: miR-153-3p, miR-5195-3p, miR-371a-3p, miR-133a-5p, miR-221-3p, miR-31-5p, miR-373-3p, miR-615-5p, miR-582-3p, and miR-301b-3p. To determine the association of these miRNAs with *PDZRN4*, PDZRN4 was measured after knocking out each miRNA respectively. It was found that, among these miRNAs, transfection with the miR-221-3p inhibitor was associated with dramatically higher levels of both protein *PDZRN4* and mRNA *PDZRN4* (*Figure 3A,B*).

To verify binding further, luciferase report assays were conducted. The results showed inhibition of luciferase activity, indicating that miR-221-3p directly binds to the 3'-UTR of *PDZRN4* mRNA (*Figure 3C*).

Further investigation revealed that, compared with normal tissues, miR-221-3p is overexpressed in colon cancer tissues (*Figure 3D*). At this point in the investigation, to confirm the presence of a regulation network, the miR-221-3p and *PDZRN4* 3'-UTR vector was transfected into 293T cells. The luciferase report assay showed that luciferase activity was dose-dependent with miR-221-3p (*Figure 3E*), and this was true also for PDZRN4 protein based on the western blot (*Figure 3F*). These results indicated the direct inhibition of *PDZRN4* expression by miR-221-3p.

To specify the binding sequence, we mutated several bases in both miR-221-3p and the *PDZRN4* 3'-UTR (*Figure 3G*). According to the dual luciferase assay, mutation in either miRNA or the *PDZRN4* 3'-UTR attenuated regulation (*Figure 3H*), and western blot was consistent with this (*Figure 3I*). These data indicated that the mutant regions of miR-221-3p and the *PDZRN4* 3'-UTR are the binding sites of this miR-3' UTR regulation system (*Figure 3G*).

Knockdown of miR-221-3p restrained tumorigenesis of HCT116 cells

To support that miR-221-3p-regulated *PDZRN4* expression interfered with the activity of colon cancer cells, miR-221-3p was knocked down by transducing HCT116 cells with a miRNA sponge. Observation and FACS detection of red fluorescent protein revealed that miR-221-3p was successfully dysregulated in HCT116 cells (*Figure 4A,B*). In addition, consistent with the results of miRNA overexpression, the levels of *PDZRN4* protein were comparable with that observed prior to knockdown of miR-221-3p (*Figure 4C*).

We then conducted more assays of phenotype. Both the cell proliferation (*Figure 4D*) and plate clone formation (*Figure 4E,F*) assays indicated that suppression of miR-221-3p expression lowered the proliferation of HCT116 cells. In addition, observations of the cell cycle showed inhibition of cell cycle progression (*Figure 4G,H*). Together, these data indicate that miR-221-3p functions in regulating HCT116 cell proliferation.

Moreover, the transwell migration, invasion, and wound-healing assays showed intense inhibition of cancer cell dissemination after treatment with the miR-221-3p sponge (*Figure 4I,J,K,L*). Together, these data indicate that miR-221-3p modulated the dissemination of colon cancer HCT116 cells.

Discussion

Gene dysregulation is a feature of cancer development. Various dysregulated genes may function as either tumor

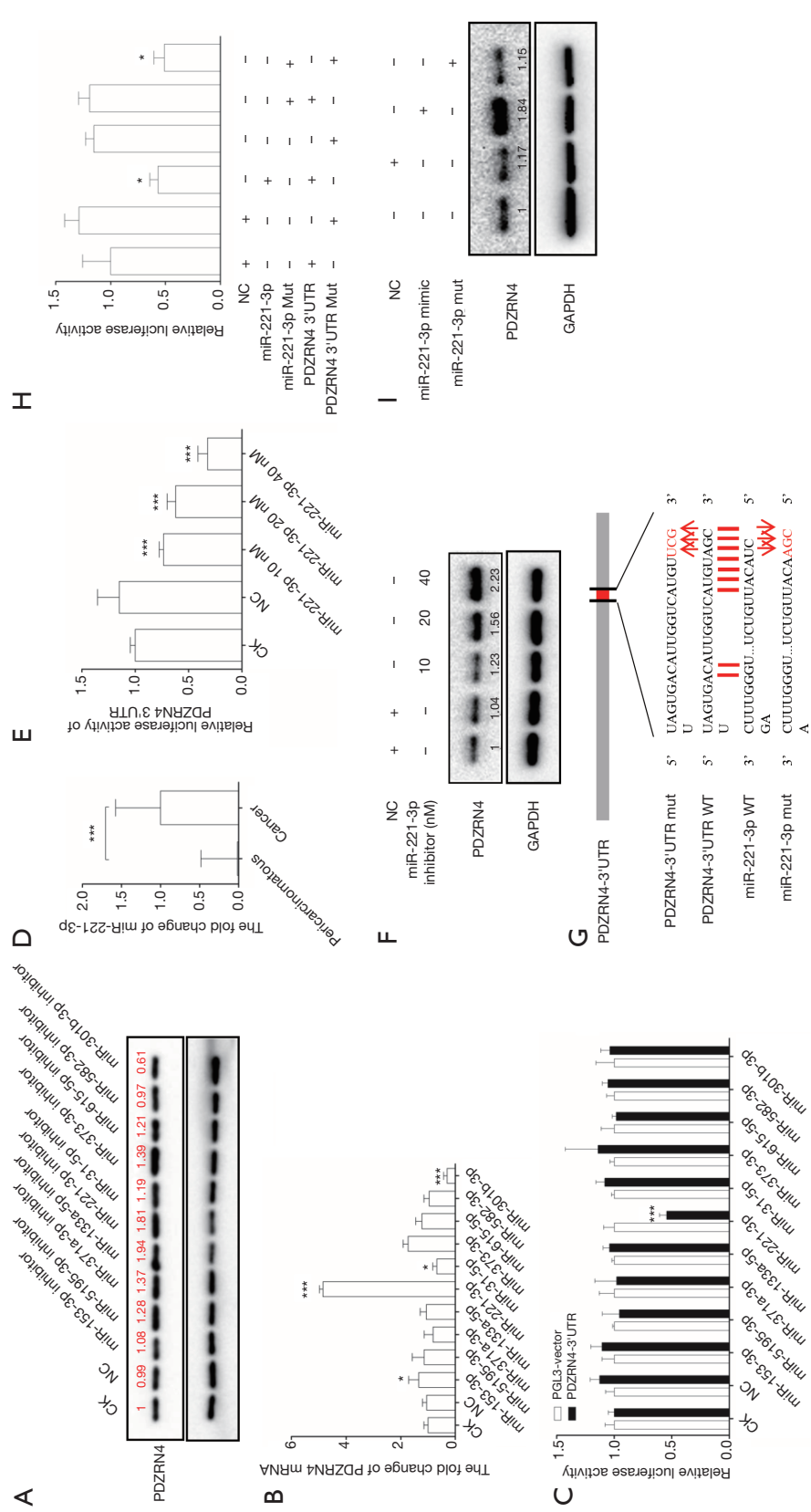
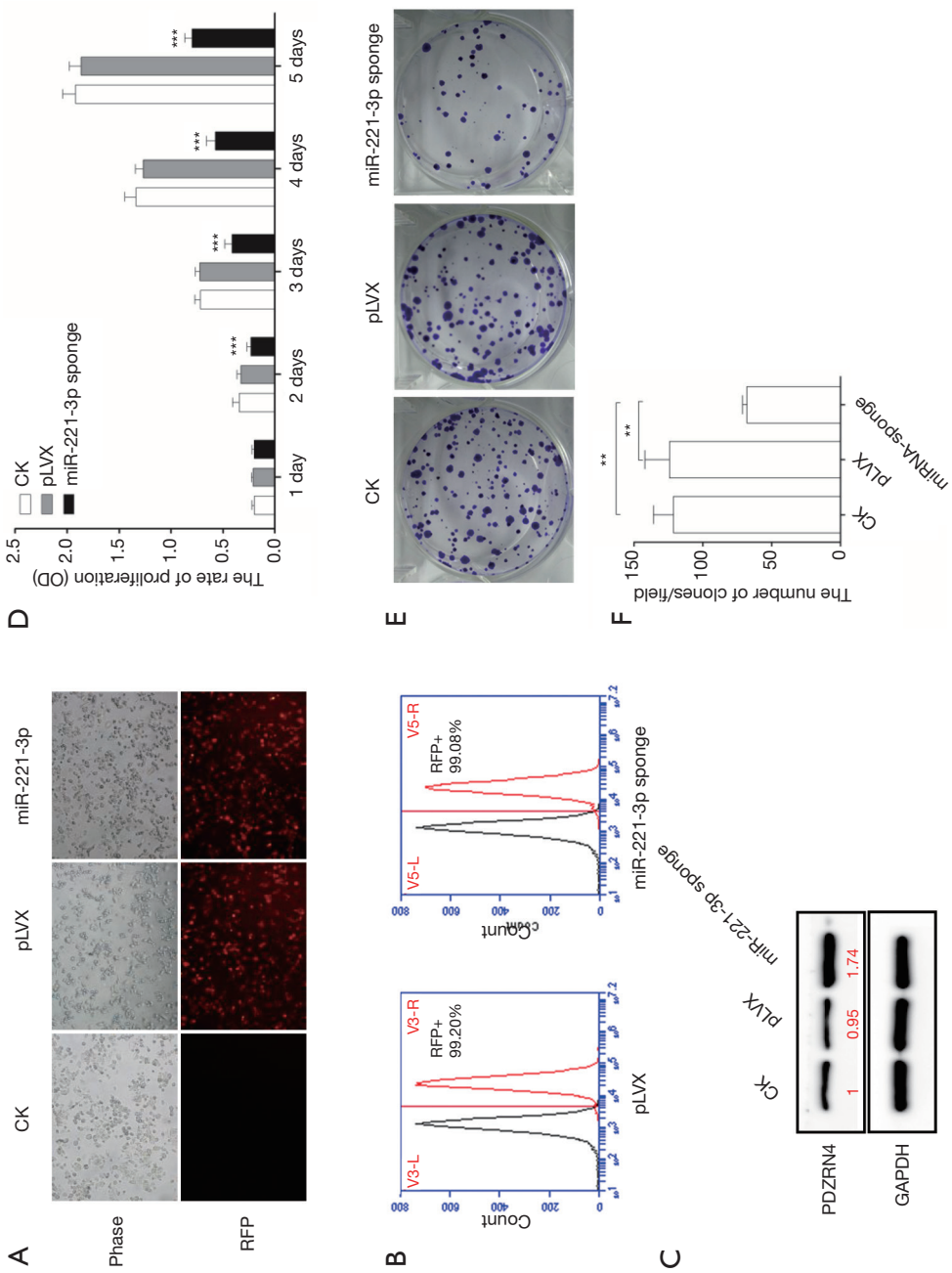


Figure 3 An overexpressed miRNA in colon cancer, miR-221-3p, directly targets PDZRN4. (A) Transfection of different miRNA inhibitors in 293T cells regulates PDZRN4 protein levels. (B) PDZRN4 mRNA in cells treated as in *Figure 3A*. *P<0.05, ***P<0.001 for Student's *t*-test. (C) Luciferase activity was detected in HCT116 cells co-transfected with different miRNAs. ***P<0.001 for Student's *t*-test. (D) miR-221-3p is upregulated in colon cancer tissues. ***P<0.001 for Student's *t*-test. (E) miR-221-3p regulated luciferase activity in a dose-dependent manner. ***P<0.001 for Student's *t*-test. (F) miR-221-3P inhibited PDZRN4 expression in a dose-dependent manner at the protein level. (G) Schematic illustration of the putative seed sequences of miR-221-3p complementary with PDZRN4 3'-UTR, and mutagenesis of binding sites in the 3'-UTR of PDZRN4 and miR-221-3p. (H) Luciferase activity was analyzed in HEK 293T cells co-transfected with miR-221-3p or miR-221-3p Mut, and PDZRN4 3'-UTR or PDZRN4 3'-UTR Mut. *P<0.05 for Student's *t*-test. (I) miR-221-3p failed to regulate endogenous PDZRN4 expression. HCT116 cells were transfected with NC, miR-221-3p mimic, or miR-221-3p mut and western blot was conducted 48 hours after transfection.



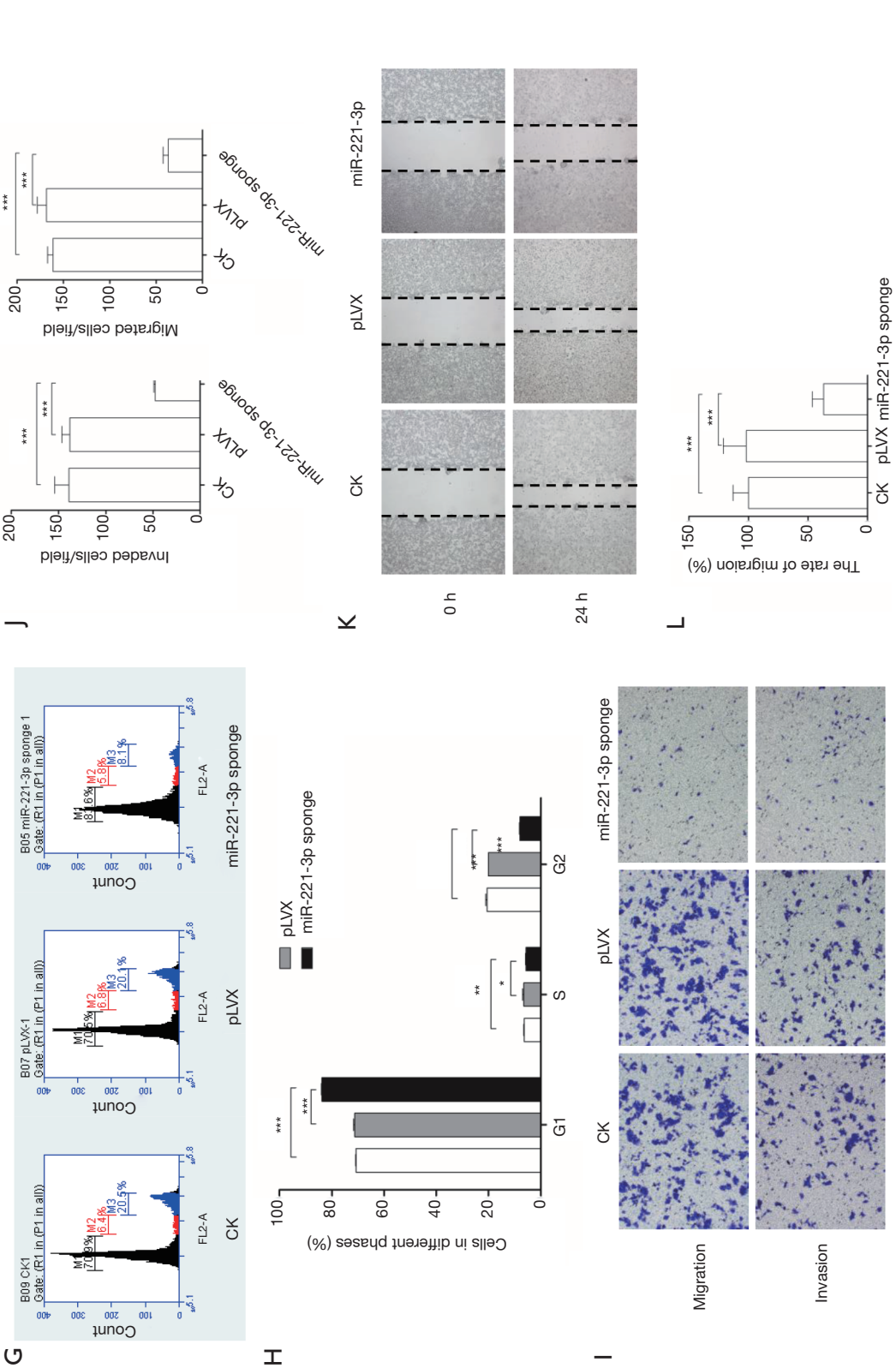


Figure 4 Knockout of miR-221-3p expression attenuated the proliferation, migration, and invasion of HCT116 cells. (A) HCT116 cells were transduced with a pLVX empty vector or miR-221-3p sponge lentivirus. Representative images of untreated cells (left) and transduced cells were taken under (Phase, top) light microscope, and (red fluorescent protein, bottom) fluorescent microscope (100×). (B) Percentage of cells treated as in *Figure 4A* tested positive for GFP by FACS. (C) PDZRN4 protein in HCT116 cells treated as in *Figure 4A*. (D) Proliferation of cells treated as *Figure 4A* by cell proliferation assay. ***P<0.001 for Student's *t*-test. (E) Representative images taken for the clone formation of cells treated as *Figure 4A*. (F) Numbers of clones formed in *Figure 4E*. **P<0.01 for Student's *t*-test. (G) Percentage of cells treated as *Figure 4A* in different cell cycle phase. (H) Percentages of cells in different cell phases. *P<0.05, **P<0.01, ***P<0.001 for Student's *t*-test. (I) Representative images for transwell migration and invasion of cells treated as *Figure 4A* (100×). (J) Number of migrated and invaded cells per field. ***P<0.001 for Student's *t*-test. (K) Representative images of wound healing assay for cells treated as *Figure 4A* (×100). (L) Percentages of wound closure. ***P<0.001 for Student's *t*-test.

suppressors or oncogenes. Thus, we searched and analyzed gene expression profiles, and chose an inhibited gene in colon cancer for further investigation, i.e., *PDZRN4*. The *PDZRN4* mRNA and PDZRN4 protein levels in colon cancer tissues were much lower than that of the adjacent normal tissues. Overexpressed *PDZRN4* in the colon cancer cell line arrested cell cycle, inhibited cell proliferation, and attenuated migration and invasion. This suggests that PDZRN4 protein in colon cancer tissues is negatively associated with cancer cell dissemination, and *PDZRN4* may function as a tumor suppressor in colon cancer development.

Furthermore, we found that *PDZRN4* translation was inhibited by an overexpressed onco-miRNA, miR-221-3p. The luciferase report assay showed a direct association between miR-221-3p and the 3'-UTR of *PDZRN4* mRNA. In addition, the cell proliferation and plate clone formation assays showed that as the miR-221-3p level subsided, so did the proliferation and dissemination of the colon cancer cell. This indicates that miR-221-3p-*PDZRN4* has an important role in regulating colon cancer development, in which miR-221-3p inhibits the translation of *PDZRN4* and thereby promotes the proliferation and dissemination of colon cancer cells.

In this study, the low number of clinical samples and time allowed limited sufficient inspection *PDZRN4* expression in colon cancer tissues. Whether *PDZRN4* protein is a viable biomarker of colon cancer requires further investigation.

Conclusions

This study investigated the expression of *PDZRN4* in colon cancer tissues, its potential function in the development of colon cancer, and its inhibition by its upstream regulator, miR-221-3p. Three main conclusions are highlighted. First, according to the gene expression profile and detection in clinical colon cancer samples, *PDZRN4* mRNA and PDZRN4 protein are downregulated in colon cancer. Second, overexpression of *PDZRN4* in the colon cancer cell line and the phenotype assays indicated that *PDZRN4* functions to attenuate cell proliferation, migration, and invasion. Thirdly, miR-211-3p inhibited *PDZRN4* translation by binding to its mRNA 3'-UTR. The knockdown of miR-211-3p dramatically reduced the proliferation, migration, and invasion of colon cancer HCT116 cells.

These discoveries suggest that *PDZRN4* may be a potential target for defeating colon cancer, but more investigations are necessary.

Acknowledgments

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.07.12). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Institutional Ethics Committee of Second Affiliated Hospital of Soochow University (Suzhou, 215004, Jiangsu Province, China) reviewed and authorized the clinical section of this research. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was taken from all patients.

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Supplementary

Table S1 Patient information enrolled in research

Patient No.	Gender	Age, years	Histological type	LNM	Tumor size, cm^3
PT 1	F	64	Mucinous adenocarcinoma	POS	3.5×4.5×1.5
PT 2	M	75	Canalicular adenoma	POS	8.0×5.5×2.5
PT 3	F	83	Signet-ring cell carcinoma	POS	7.0×4.5×2.0
PT 4	M	79	Mucinous adenocarcinoma	POS	5.0×4.0×2.3
PT 5	M	63	Canalicular adenoma	POS	3.5×2.0×1.0
PT 6	F	37	Canalicular adenoma	POS	5.0×4.5×2.0
PT 7	F	85	Canalicular adenoma	NEG	4.0×4.0×1.0
PT 8	M	51	Signet-ring cell carcinoma	POS	6.0×2.5×2.0
PT 9	F	84	Canalicular adenoma	NEG	10.0×5.0×4.5
PT 10	M	73	Canalicular adenoma	NEG	5.5×3.5×2.0
PT 11	M	74	Canalicular adenoma	POS	4.0×3.3×1.5
PT 12	F	55	Canalicular adenoma	NEG	1.1×0.9×0.5
PT 13	F	58	Canalicular adenoma	POS	6.5×4.0×3.0
PT 14	F	74	Mucinous adenocarcinoma	POS	3.5×2.0×1.5
PT 15	M	64	Polypoid adenocarcinoma	POS	3.0×2.2×1.6
PT 16	M	60	Canalicular adenoma	POS	4.3×3.2×1.5
PT 17	F	70	Canalicular adenoma	POS	8.0×5.0×2.5
PT 18	F	58	Canalicular adenoma	POS	6.0×3.5×2.0
PT 19	M	71	Canalicular adenoma	POS	3.2×3.0×0.8
PT 20	M	78	Canalicular adenoma	POS	6.0×4.0×4.0

F, female; LNM, lymph node metastasis; M, male; NEG, negative; POS, positive; PT, patient.

VIP	*PPP1R14A*	*ADAMTS8*	*CNRIP1*	*PTN*	*CCL21*
RERGL	*MFAP5*	*PPP2R2B*	*FERMT2*	*SGCE*	*FGL2*
MYH11	*PTGS1*	*NEXN*	*DDR2*	*ANTXR2*	*KCNMB1*
DES	*SPARCL1*	*MEIS1*	*MEIS2*	*ANGPTL1*	*PDE5A*
C7	*LMOD1*	*SYNC*	*PRUNE2*	*HSPB3*	*SPEG*
PGM5	*LPAR1*	*SYT4*	*PODN*	*TSHZ3*	*GCNT2*
CNN1	*STMN2*	*GPM6A*	*PDLIM3*	*FCER1A*	*AFF3*
MYOM1	*RUNDC3B*	*ADHFE1*	*SGCA*	*SMARCD3*	*GNAO1*
CFD	*STOX2*	*JAM2*	*ATP2B4*	*LMOD3*	*PTCHD1*
RBPMS2	*CDH19*	*SMYD1*	*RERG*	*EIF4E3*	*POPDC2*
ATP1A2	*MYOT*	*SLC9A9*	*GREM2*	*TBC1D9*	*SMTN*
FHL1	*RGMA*	*PRRT2*	*EBF1*	*ANKS1B*	*MMRN1*
CLEC3B	*SETBP1*	*FOSB*	*CASZ1*	*ARMCX1*	*TAGLN*
MAMDC2	*ACACB*	*AHNAK*	*PLN*	*CAP2*	*TACR2*
ABCA8	*DACT3*	*SLC25A23*	*TAGLN3*	*MRGPRF*	*CYBRD1*
DPT	*SRPX*	*SLIT2*	*ELAVL4*	*BNC2*	*ANK2*
NPTX1	*FXYD6*	*LYVE1*	*MAF*	*SLC6A16*	*P2RX1*
PRPH	*GNG7*	*TEF*	*MASP1*	*RPRM*	*COLEC12*
SCARA5	*LIMS2*	*PLEKHO1*	*HSPA2*	*LRRFIP1*	*TMOD1*
HAND1	*CD209*	*CITED2*	*KCNN3*	*ARRDC4*	*CACNA1H*
RNF150	*NNAT*	*CNTFR*	*ETFDH*	*REEP1*	*MXI1*
CASQ2	*GSN*	*PALM*	*MAP1B*	*FMN2*	*GPR162*
HSPB6	*PLP1*	*ZSCAN18*	*EMP3*	*OPTN*	*JPH2*
PLAC9	*ADH1B*	*SCN9A*	*AGTR1*	*LDB3*	*SLIT3*
CXCL12	*SCG2*	*PCP4*	*ADAMTS1*	*OLFML1*	*FOLR2*
ADH1A	*PI16*	*CBX7*	*TSPAN18*	*ZNF671*	*SLC22A17*
LRRN2	*FNBP1*	*PINK1*	*KIAA1683*	*ADAMTSL3*	*A2M*
FAM107A	*PCOLCE2*	*PDZD4*	*CSF1R*	*VIPR2*	*ADCY9*
CKB	*FLNA*	*BCHE*	*STBD1*	*SCUBE2*	*TPM1*
PDE9A	*PDE7B*	*NLGN1*	*REEP2*	*PMP22*	*CBX6*
ACTG2	*FXYD1*	*SORBS2*	*OLFML2A*	*GFRA3*	*MAOB*
SEMA6A	*IGFBP6*	*HTR4*	*SPECC1L*	*NGB*	*DIXDC1*
MYLK	*FOXF2*	*PDZRN4*	*GSTM2*	*LGI4*	*RASGRP2*
SCGN	*TSC22D3*	*KIAA0513*	*AXL*	*SYNGR1*	*UCHL1*
C2orf40	*TMEM100*	*TCN2*	*GSTM5*	*TGFB1I1*	*DENND5B*
PNCK	*PEG3*	*SNAP91*	*ARHGEF37*	*SALL2*	*CELF2*
PDK4	*SORBS1*	*GLIPR2*	*RGL1*	*DAAM2*	*RCSD1*
GPX3	*THBS4*	*MS4A7*	*MYOC*	*LINC00341*	*CSPG4*
CRYAB	*CLIP3*	*RELN*	*GLI3*	*DSEL*	*CLMP*
HSPB8	*MGP*	*PTH1R*	*FAM189A2*	*ROR2*	*CBLN2*
NPY	*STAB1*	*KCNMA1*	*F13A1*	*KCNH2*	*EML1*
METTL7A	*XKR4*	*TGFBR3*	*LRCH2*	*PPP2R3A*	*DDAH2*
TCEAL2	*KIF1A*	*BOC*	*KIT*	*CHODL*	*LPP*
ABI3BP	*LIFR*	*PSG11*	*TRIM9*	*SGCD*	*CHST15*
MFAP4	*FAM46B*	*PDGFRA*	*PTRF*	*FGF13*	*KCNIP3*
GFRA2	*CPXM2*	*KANK2*	*SSBP2*	*CCDC136*	*SNAP25*
ZBTB16	*CSRP1*	*MEF2C*	*TNFSF12*	*ANKRD35*	*GYPC*
SDPR	*COX7A1*	*C15orf52*	*KCNA5*	*SOBP*	*OGN*
GABARAPL1					

Figure S1 Upregulated genes in both arrays (by acronym).

SLC38A5	*CNPY2*	*KNTC1*	*JPH1*	*HSPD1*	*PSAT1*
DACH1	*ANKRD22*	*UBD*	*TIMELESS*	*CCNO*	*HMMR*
CHPF	*TOP1MT*	*FBL*	*MCM7*	*EEF1E1*	*PUS7*
OLR1	*KPNA2*	*KCTD14*	*HSPH1*	*SLC6A14*	*CCNB2*
BLM	*C4BPB*	*EZH2*	*FAM84B*	*TROAP*	*ITGA2*
NCAPD2	*EPHB2*	*CFB*	*CST1*	*GNL3*	*KIAA0101*
NIPSNAP1	*RANGAP1*	*CDCA8*	*SULT2B1*	*ASF1B*	*TESC*
GJB3	*POLE2*	*GSTO2*	*HES6*	*MYEOV*	*NCAPG*
RECQL4	*CECR5*	*KIF14*	*IL24*	*SALL4*	*AURKA*
DARS2	*C10orf2*	*SPAG5*	*NUP37*	*MMP10*	*NME1*
RHBDF2	*TMEM63A*	*METTL1*	*CDT1*	*RPS21*	*NUSAP1*
AZGP1	*LIPG*	*SH3BP4*	*CENPE*	*GDF15*	*TMEM97*
RFWD3	*KLHL35*	*NOP16*	*EBPL*	*LMNB2*	*MCM4*
SPC25	*MCM10*	*NUP62CL*	*SHMT2*	*LRP8*	*UBE2T*
BRCA1	*HPRT1*	*SKA3*	*EPHB3*	*TYMS*	*CENPN*
GGCT	*E2F7*	*DDIT4*	*MET*	*RAD51AP1*	*CENPF*
DUSP4	*FOXM1*	*NDC80*	*POLQ*	*TK1*	*GINS2*
ICA1	*CENPK*	*ZWINT*	*GPT2*	*PAQR4*	*MAD2L1*
CXCL10	*NUDT5*	*RFC5*	*KIF4A*	*MCM2*	*TRIB3*
GMNN	*KCNN4*	*MND1*	*RTKN*	*CELSR3*	*SCD*
ZNF593	*PAFAH1B3*	*MLXIPL*	*KIF11*	*PBK*	*PAICS*
MRPS12	*HMGB3*	*ANAPC1*	*IL17RB*	*SPINK1*	*CTHRC1*
GPX2	*EARS2*	*CHTF18*	*DHCR7*	*C19orf48*	*PTTG1*
SRPRB	*FAM64A*	*KLK6*	*CKAP2L*	*TEAD4*	*IQGAP3*
MIPEP	*MMP12*	*IFITM1*	*PCNA*	*MMP11*	*SERPINB5*
NOB1	*ATIC*	*BYSL*	*AURKB*	*ENC1*	*KRT80*
GMDS	*BORA*	*WDR4*	*FERMT1*	*MELK*	*CLDN2*
SQLE	*YARS2*	*PODXL2*	*KIF2C*	*CENPA*	*TRIP13*
DIO2	*SF3B3*	*STIL*	*MARCKSL1*	*CENPW*	*SOX9*
KIF23	*TMPRSS3*	*FANCD2*	*TGIF1*	*BUB1B*	*TPX2*
PAM16	*IL1RN*	*PLEK2*	*RAD54L*	*PYCR1*	*PRC1*
RPIA	*RCC2*	*REEP6*	*FANCI*	*NEBL*	*CKS2*
SLC1A5	*SRPK1*	*ECE2*	*CBX2*	*S100P*	*CDC20*
TGDS	*TSFM*	*SLC25A15*	*S100A11*	*RNF43*	*CEP55*
RAD54B	*CDC45*	*MTHFD2*	*KIF15*	*KRT6B*	*ASPM*
SLC16A10	*FAM57A*	*GART*	*POLR1C*	*KIF20A*	*SLCO4A1*
CHD7	*SYBU*	*OLFM4*	*GGH*	*DKC1*	*CDH3*
NCAPD3	*RUVBL2*	*PLAU*	*IMPDH2*	*CCNB1*	*REG1B*
BACE2	*SLC12A8*	*CMTM8*	*HJURP*	*NFE2L3*	*CXCL1*
TBRG4	*E2F5*	*RRP9*	*PPAT*	*HOXB8*	*TOP2A*
KRTCAP3	*SLC25A22*	*NUF2*	*PRDX4*	*BUB1*	*CDCA7*
MTHFD1	*CCNF*	*GNPNAT1*	*EXO1*	*CCNA2*	*ASCL2*
AEN	*FUT4*	*RMI2*	*SNRPF*	*CDCA3*	*MMP1*
FBXO5	*ZDHHC9*	*SLC29A2*	*PUS1*	*DLGAP5*	*REG1A*
FABP6	*C16orf59*	*FOXA2*	*WDR12*	*DPEP1*	*CLDN1*
OIP5	*NOP56*	*KLK11*	*TOMM34*	*TTK*	*MMP7*
RPA3	*CHAF1B*	*CDC25A*	*FASN*	*SLC12A2*	*FOXQ1*
PKMYT1	*F12*	*RAB15*	*SOX4*	*CDKN3*	*UHRF1*
IRX2	*FAM60A*	*PLK4*	*LCN2*		

Figure S2 Downregulated genes in both arrays.