Original Research

Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metallopeptidase 9 transcriptional activity

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Abstract

Piwi-like protein 2 (Piwil2) has recently emerged as a putative oncogene which is amplified in several human malignancies. However, the role of Piwil2 in colon cancer remains poorly understood. The aim of this study was to investigate the clinical and pathological significance of Piwil2, and the possible role in the proliferation and metastasis of colon cancer. Primary colon cancer paired with adjacent normal colon tissue and lymph node metastasis (LNM) lesions in 66 patients' tissue microarrays (TMA) were used to determine the expression of Piwil2. Knocked down Piwil2 expression in SW620 and SW480 colon cancer cell lines was performed to evaluate the role of Piwil2 in cell proliferation, invasion, metastasis *in vitro* and tumorigenecity *in vivo*. The possible roles of Piwil2 in the regulation of a 2 kb matrix metallopeptidase 9 (MMP9) promoter fragment and on the regulation of apoptotic pathways were evaluated by using a luciferase reporter construct and Western blots, respectively. Significantly higher expression levels of Piwil2 were observed in primary colon cancer tissue and in LNM in comparison with normal colon mucosa. Piwil2 expression significantly correlated with more aggressive clinical and pathological parameters with poorer five-year metastasis-free survival and overall survival. Piwil2 silencing significantly reduced cancer cell proliferation, colony formation ability and increased apoptosis *in vitro* and inhibited tumor growth *in vivo*. Piwil2 knockdown also attenuated migration and invasion of colon cancer cells via modulation of MMP9 transcriptional activities. Our results indicate that Piwil2 moderates the proliferation and metastasis potential of colon cancer.

Keywords: colon cancer, metastasis, MMP9

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Introduction

Recent cancer stem cell theories propose that cancer develops from a subset of malignant cells which possess stem cell characteristics and account for the initiation and progression of a variety of malignancies, including colon cancer.[1] These cancer stem cells (CSC) possess characteristics of both stem cells and cancer cells, such as self-renewal, asymmetric cell division, resistance to apoptosis, tumorigenicity and metastatic potential.[2] Furthermore, stem and progenitor cells are of great interest in cancer research because it is believed that these cells are the initial targets for both malignant transformation and onset of cancer.[3] The study of stem cell self-renewal genes has great potential to expand our understanding of carcinogenesis and to develop novel strategies for preventing and treating cancers.[4]

Piwil2 is a member of the P-element-induced wimpy testis/Argonaute (PIWI/AGO) gene subfamily, the members of which are characterized by conserved PAZ and PIWI domains and represent the first class of genes known to be required for stem-cell self-renewal in diverse organisms.[5,6] In humans, the Piwi gene family includes Piwil1, Piwil2, Piwil3 and Piwil4, which all play crucial roles in RNA silencing, spermatogenesis, gametogenesis and translational regulation.[7,8] Suppressing Piwi expression in single germline stem cells significantly decreased the rate of their division, while Piwi overexpression in somatic cells caused an increase both in the number of germline stem cells and the rate of their division.[5] Interestingly, Piwil2 was recently shown to be stably expressed in precancerous stem cells (pCSCs), which have the potential for either benign or malignant differentiation, and Piwil2 is thought to regulate pCSC development and differentiation.[9]

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Since overexpression of the Piwi genes could lead to disturbance of cell division and cause malignant transformation, the Piwi genes are thought to be putative proto-oncogenes.[10] Piwil1 was reported to be overexpressed during the progression of human esophageal squamous cell carcinoma and gastric cancer.[11,12] Although Piwil2 is mainly expressed in the testis and embryonic cells in normal tissue, it is widely expressed in a number of tumors.[13] Piwil2 was recently shown to be expressed at various stages of human cervical squamous cell carcinomas and adenocarcinomas, suggesting that it could be a useful marker for early detection of cervical cancer.[14] Piwil2 mRNA and protein levels are also upregulated in testicular germ cell tumors, where Piwil2 inhibited apoptosis through activation of the Stat3/Bcl-XL pathway.[10] Overexpression of Piwil2 at various stages of breast cancer suggests that it can be used as a novel biomarker for breast cancer.[15] Interestingly, Piwil2 was not present in human bladder cancer cell lines or tissues, suggesting that it did not play a role in carcinogenesis of bladder cancer.[4]

However, the role of Piwi2 in the pathogenesis and progression of colon cancer remains unclear. The central focus of this study was to investigate the clinical and pathological significance of Piwil2 and the underlying mechanism of the possible potential role in the proliferation and metastasis of colon cancer.

Materials and methods

Clinical samples

A total of 203 patients with colon cancer underwent surgery by the same surgical team at the General Surgery Department of Shanghai JiaoTong University Affiliated First People's Hospital between 2001 and 2003. None of these patients received either chemotherapy or radiotherapy prior to surgery. There were 86 men and 117 women with a median age of 68 y (range, 22–95 y). We included 66 cases of primary colon cancer paired with adjacent normal colon tissue and lymph node metastasis (LNM) lesions in our tissue microarray (TMA). Staging was based on pathological findings according to the American Joint Committee on Cancer (AJCC). Based on the tumor (T), node (N), metastasis (M) classification system, we identified 24 cases at stage I, 81 cases at stage II, 80 cases at stage III and 18 cases at stage IV. All patients provided informed consent and the protocol was approved by the Institutional Review Board of Shanghai First People's Hospital.

TMA construction and immunohistochemistry

Tissue array construction was performed as described previously.[16] Sections (4 μm thick) of TMA slides were prepared and processed for immunostaining using a 1:50 dilution of a rabbit polyclonal antibody against human Piwil2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with the Envision kit (Dako Cytomation, Glostrup, Denmark), according to the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded samples were subjected to pressure cooker-mediated antigen retrieval in citrate buffer (pH 6.0) for seven minutes. Sections were incubated with 1:50 dilution of anti-Piwil2 overnight at 4°C and then incubated with goat anti-rabbit Envision System Plus-HRP for 30 min at room temperature. Sections were rinsed three times in phosphate-buffered saline for 10 min each, incubated with diaminobenzidine for one minute, counterstained with Mayer haematoxylin, dehydrated and mounted.

Immunoreactivity was evaluated independently by two researchers in a blinded fashion. The evaluation was based on the staining intensity and extent of staining. Staining intensity was graded as follows: 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+, intense staining. The staining area was scored using the following scale: 0, no staining of cells; 1+, <10% of tissue stained positive; 2+, 10–50% stained positive; and 3+, >50% stained positive. The sum of staining score (intensity + extension) index was designated as follows: 0–2, negative expression; 3–4, weak expression; and 5–6, strong expression.

Cell culture and reagents

Human colon cancer cell lines SW620 and SW480 were all cultured at 37°C, high humidity and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% streptomycin and penicillin.

siRNA/shRNA transfection

The target sequences of the Piwil2 gene siRNA were (siRNA#1: 5′ AAAGCCAGAGGAACCAAGCAC 3′) and (siRNA#2: 5′ AAACCTTTGGACCCAGCTCTG 3′). A scrambled siRNA (5′ GTACCGCACGTCATTCGTATC 3′) that had no homology with mammalian mRNA sequences was used as a negative control. Cells were transfected with siRNAS using Oligofectamine (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

For generation of stable clones, the specific siRNA target sequence was cloned in the pSilencer^{TM} 2.1-U6 puro Vector (Ambion, Grand Island, NY, USA) between the BamHI and HindIII restriction sites according to the manufacturer's protocol. Cells were transfected with siRNA vectors using Lipofectamine TM2000 (Invitrogen) according to the manufacturer's instructions. Stable colonies were selected in the presence of 10 μg/mL puromycin (Sigma, St Louis, MO, USA) supplemented with growth medium. After six weeks, the individual positive clones were expanded, and the inhibitory effect was confirmed by Western blot analysis.

Cell proliferation assay

For proliferation assays, 96-well plates were seeded with each group of cells at a density of 1500 cells per well and cultured for 24, 48, 72 or 96 h, respectively. The cells were then incubated for two hours with 100 μL of 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in DMEM). The culture medium was removed and 200 μL dimethyl sulfoxide was added to dissolve the formazan. Finally, absorbance at 570 nm was measured to determine cell viability. The cell viability of different groups at each measuring time point was compared.

Soft agar assay

Cells were suspended in 0.3% agar medium (DMEM containing 10% FBS) and then plated on a 0.6% agar base layer at a concentration of 1000 cells per six-well plate. The cells were incubated in a humidified atmosphere (5% CO_2) at 37°C. The number of colonies that were 50 μm or larger were counted after 10 days.

Tumorgenicity assay

Six-week-old athymic nude mice were subcutaneously injected in the flank with SW620 cancer cells stably transfected with sh-Piwil2 or sh-control vectors (1×10^6 cells; $n = 5$–7 mice per group) as previously described.[17] Tumor volume and body weight of the mice were measured once in a week and mice were sacrificed after 36 days. All procedures involving mice were conducted in accordance with Shanghai Jiaotong University Affliated Shanghai First People's Hospital Animal Care guidelines. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize possible alternatives to *in vivo* techniques.

In vitro migration and invasion assays

The migration and invasion assays were conducted in a modified 24-well Boyden chamber with uncoated membranes or membranes coated with Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, 500 μL of a SW620 or SW480 cell suspension (8×10^4) prepared in serum-free medium was loaded in the upper well of the chamber, while medium containing 10% FBS was placed in the lower wells as a chemoattractant stimulus. The chamber was incubated for 24 or 48 h, and the non-migratory and non-invasive cells in the upper chamber of the filter were removed with a cotton swab. The migrated cells on the bottom surface of the filter were fixed, stained and counted under a microscope in four randomly selected fields at $\times$200 magnification.

Realtime reverse transcription-polymerase chain reaction

mRNA levels of the matrix metallopeptidase 9 (MMP9) gene were determined by realtime reverse transcription-polymerase chain reaction (PCR) analysis with SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Each sample was run in triplicate for the target gene and the internal control gene.

Western blot analysis

Cellular protein was extracted from cultured cells and tissue using the Whole Protein Extraction Kit (Fermentas, Rockford, IL, USA). Cell lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto poly(vinylidene difluoride) membranes. Membranes were blocked with 5% non-fat dry milk in TBST (Tris buffer solution with Tween) buffer and probed with primary antibody Piwil2 (Epitomics, Burlingame, CA, USA; 1:500 dilution), MMP9 (Santa Cruz Biotechnology; 1:500 dilution), caspase-3 (Cell Signaling, Danvers, MA, USA; 1:500 dilution) and poly(ADP–ribose) polymerase (PARP) (Santa Cruz Biotechnology; 1:500 dilution) at 4°C for overnight, followed by a secondary antibody-horseradish peroxidase conjugate (Cell Signaling; 1:2000 dilution) at room temperature for one hour. Protein signals were detected using chemiluminesence (Pierce, Rockford, IL, USA) and autoradiography.

Zymography

MMP9 activity was detected by zymography as described previously.[18] Briefly, 10 μL of the medium from the serum-free Piwil2 knockdown group and control groups was mixed with the same volume of sample buffer and applied to 8% (wt/vol) polyacrylamide gels containing 1 mg/mL gelatin. Gels were incubated in 2.5% Triton X-100 for 45 min after electrophoresis, then incubated at 37°C overnight in 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $CaCl_2$, 1 mmol/L $ZnCl_2$ and 0.02% NaN_3, followed by staining with 0.1% Coomassie brilliant blue.

Promoter reporters and dual-luciferase assay

We used the MMP9 promoter to drive expression of a luciferase reporter as previously described.[16] Briefly, the 5′ region (−1900 to +150, relative to the transcription start site) of the Piwil2 gene was amplified by PCR using sense primer 5′ GGGGTACCCCGCCCAAGGTCACAT 3′ and antisense primer 5′ CCAAGCTTGGCAGGGAAGAGCACA 3′. This 2 kb fragment was inserted into the pGL3-Basic Reporter Vector (Promega, Madison, WI, USA) between the KpnI and HindIII restriction sites. Transfection efficiency was normalized by co-transfection with a phosphorylated β-actin-Renilla luciferase reporter containing a full-length Renilla luciferase gene. We quantified both firefly and Renilla luciferase activity using a dual-luciferase assay system (Promega) 48 h after transfection.

Statistical analysis

The analysis of variance test was used to determine the statistical significance of differences between experimental groups. The Kaplan–Meier method was used to analyze colon cancer patients' cumulative survival rates. A Cox proportional hazards model was used to calculate univariate and multivariate hazard ratios for the study variables. SPSS software 13.0 (SPSS, Chicago, IL, USA) was used for the analyses. A P value of <0.05 was considered as statistically significant.

Results

Aberrant overexpression of Piwil2 in colon cancer tissue

We evaluated the potential role of Piwil2 in colon cancer by using Western blots to analyze Piwil2 protein expression in primary colon cancerous tissue and paired normal colonic mucosa. It showed that Piwil2 was highly expressed in cancerous tissue relative to non-cancerous mucosa in eight cases of colon cancer patients' specimens (Figure 1a).

Expression and subcellular localization of Piwil2 protein was determined by immunohistochemistry in a TMA containing 203 cases of primary colon cancer paired with its non-cancerous tissue and paired 66 LNM lesions. We showed Piwil2 overexpression in 74.9% (152/203) of primary cancer lesions. Primary cancer lesions had a significantly higher degree of staining compared with adjacent normal colonic tissue, which exhibited only negative or weak positive staining in 8.8% (18/203) of cases (Figure 1b). As shown in Figure 1c, Piwil2 protein was mainly localized in the cytoplasm of cancer cells. It is worth noting that strong Piwil2 staining was observed in metastatic colon cancer cells within positive lymph nodes. In the 66 matched specimens available for analysis, the rate of positive Piwil2 expression in colon cancer LNM cells was higher than in the

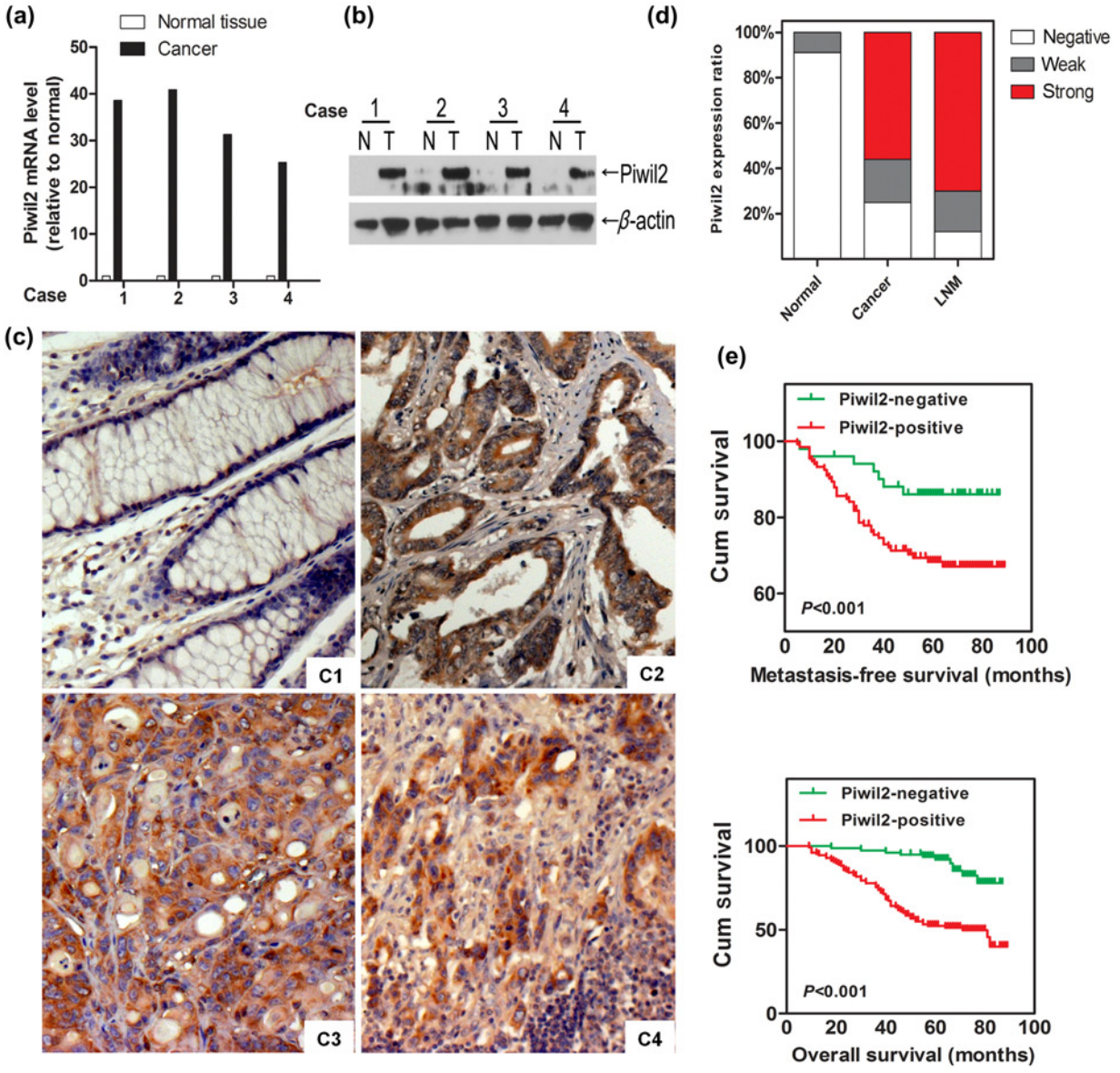

Figure 1 Analysis of Piwil2 expression in colon cancer tissues. (a) Western blotting analysis was performed to examine Piwil2 expression in eight cases of primary colon carcinomas (T) and paired non-cancerous adjacent tissues (N). (b) Schematic diagram of Piwil2 expression in 203 cases of paired normal colon tissue, primary colon cancer and 66 cases of LNM. Piwil2 expression was significantly higher in LNM tissue compared with primary colon cancer and normal colon tissue ($P < 0.001$). (c) Immunohistochemical staining for Piwil2 in TMA. Piwil2 was localized within the cytoplasm. Left panel: elevated Piwil2 expression in the tumor cells of colon cancer tissue (*T*) compared with those of adjacent normal mucosa with negative staining (*N*). (d) The metastasis-free survival (MFS) and overall survival (OS) rates were estimated by the Kaplan–Meier method. Both the MFS rate and OS rate of patients with Piwil2-positive primary tumors was significantly lower than that of patients with Piwil2-negative primary tumors (log-rank test, $P < 0.001$). LNM, lymph node metastasis; TMA, tissue microarray. (A color version of this figure is available in the online journal)

Table 1 Correlation between Piwil2 expression and clinicopathological characteristics

	Cases	Piwil2 protein expression (n)			*P* value
		Negative (51)	Weak (39)	Strong (113)	
Age					
<65	81	17	21	43	0.12
≥65	122	34	18	70	
Gender					
Male	86	27	15	44	0.21
Female	117	24	24	69	
Location					
Right	84	20	18	46	0.988
Transverse	19	6	3	10	
Left	20	5	4	11	
Sigmoid	80	20	14	46	
Stage					
I	24	8	7	9	0.006
II	81	19	20	42	
III	80	24	11	45	
IV	18	0	1	17	
T stage					
T1	8	2	3	3	0.025
T2	23	6	7	10	
T3	76	27	14	35	
T4	96	16	15	65	
N stage					
N0	108	27	28	53	0.035
N1	61	19	6	36	
N2	34	5	5	24	
Metastasis					
M0	185	51	38	96	0.002
M1	18	0	1	17	
Vessel invasion					
No	189	46	38	105	0.403
Yes	14	5	1	8	
Differentiation					
Well	99	28	23	48	0.294
Moderate	74	16	10	48	
Poor	30	7	6	17	

paired primary tumors (58/66, 87.9% versus 46/66, 69.7%; $P = 0.001$).

The relationship between clinical and pathological characteristics and Piwil2 expression in 203 patients with colon cancer in our TMA is summarized in Table 1. We observed that the levels of Piwil2 expression were closely correlated with the T classification (T1–T2 versus T3–T4; $P = 0.025$), lymph node involvement (N classification, N0–N1 versus N2–N3; $P = 0.035$), distant metastasis (no versus yes; $P = 0.002$) and clinical stage (I–II versus III–IV; $P = 0.006$) in colon cancer patients. Taken together, these findings further indicate that Piwil2 may be involved in carcinogenesis and metastasis of colon cancer.

High Piwil2 expression is associated with poor clinical outcome in human colon cancer

To analyze the prognostic value of Piwil2 for distant metastasis, we specifically assessed Piwil2 staining in 185 patients at stage I–III who accepted radical colectomy. There was a significant difference between the Piwil2-positive and Piwil2-negative groups in the proportion of patients who developed metastasis from the primary tumor after radical colectomy (Figure 1d). The risk of developing distant metastases after radical colectomy was much higher in patients with Piwil2-positive tumors (41 [30.6%] of 134 patients, mean follow-up 68.9 months [range 63.7–74.1]) compared with patients with Piwil2-negative tumors (7 [13.7%] of 51, mean follow-up 79.0 months [range 73.3–84.7]) ($P = 0.015$). Piwil2 could serve as a novel prognostic marker to predict risk of distant metastases in patients with radical colectomy (hazard ratio [HR] 2.63; 95% confidence interval [CI] 1.17–5.81; $P = 0.019$).

Kaplan–Meier analysis also showed that the expression of Piwil2 was significantly correlated with overall survival (OS) of colon cancer patients (log-rank test, $P < 0.001$, Figure 1d). Piwil2-positive patients had a significantly lower five-year OS rate than those with Piwil2-negative tumors (56.6% versus 84.3%, HR 3.31; 95% CI 1.59–6.9; $P = 0.001$). In multivariate analysis with clinicopathological variables, including clinical stage, differentiation grade and venous invasion, the expression of Piwil2 had an independent prognostic value to predict patients' outcomes (Table 2).

Reduced Piwil2 expression suppressed the proliferation and induced apoptosis of colon cancer cells both *in vitro* and *in vivo*

To investigate the biological function of Piwil2, we used two siRNAs (Si-Piwil2#1 and Si-Piwil2#2) specifically targeting Piwil2, to transiently knock down the endogenous expression of Piwil2 in SW620 cells, a colon cancer cell line with high metastatic behavior. Western blotting data showed that Si-Piwil2#1 more effectively reduced the

Table 2 Multivariate analysis of metastasis-free survival (MFS) and overall survival (OS) of 203 colon cancer patients

Variable	Multivariate analysis (MFS)			Multivariate analysis (OS)		
	P value	HR	CI (95%)	*P* value	HR	CI (95%)
AJCC Stage (I/II versus III/IV)	<0.001	4.95	2.5–9.84	<0.001	5.59	3.07–10.15
Histologically poorer grade*	N.S.	1.89	0.85–4.2	<0.001	3.15	1.79–5.54
Vessel invasion (no versus yes)	0.008	3.43	1.37–8.58	N.S.	1.98	0.99–3.99
Piwil2 (negative versus positive)	0.001	4.03	1.74–9.33	<0.001	4.33	2.05–9.13

AJCC, American Joint Committee on Cancer; NS, not significant; HR, hazard ratio; CI, confidence interval

*'Histologically poorer grade' corresponded to signet ring cell carcinoma, mucinous adenocarcinoma and poorly differentiated adenocarcinoma

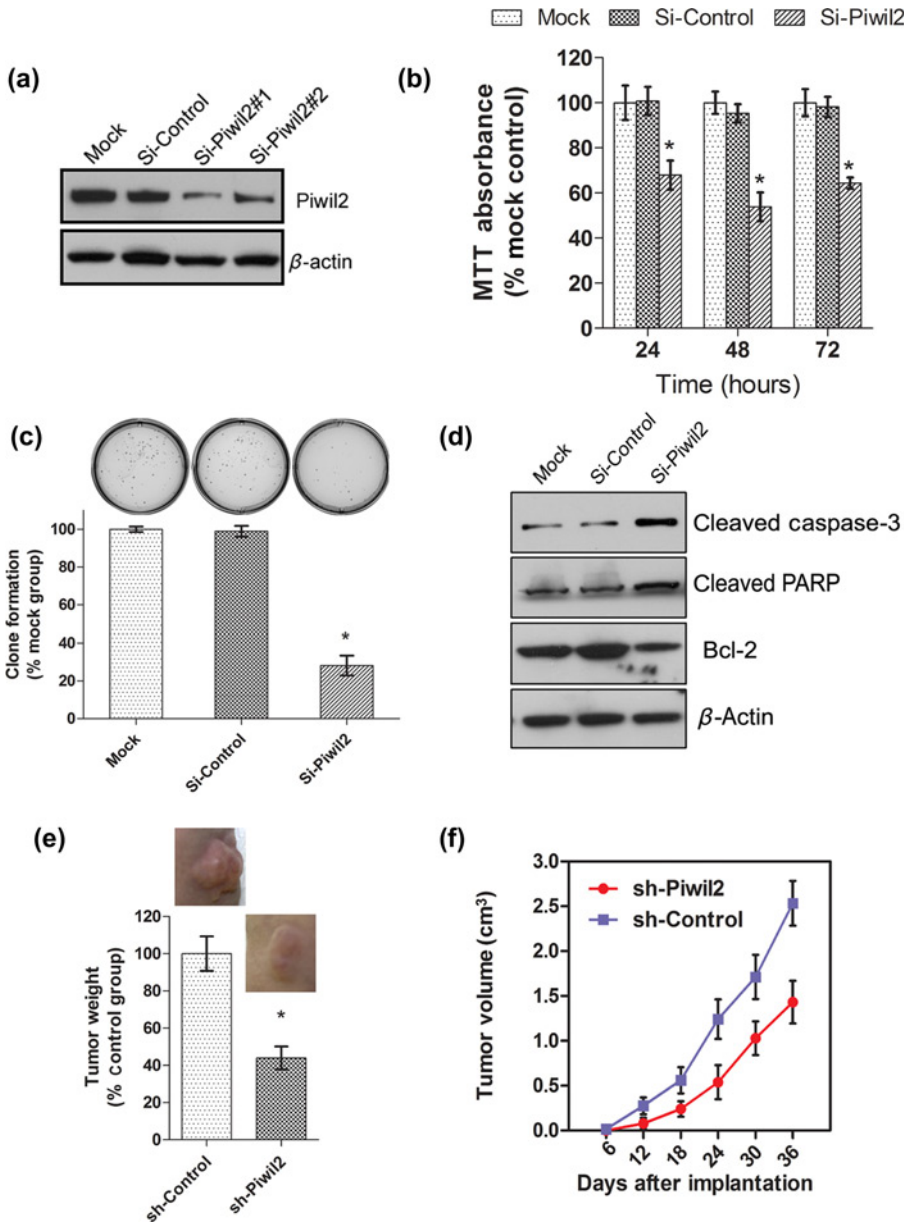

Figure 2 Effects of Piwil2 knockdown on the growth of SW620 cells. (a) SW620 cells were transfected with Piwil2 siRNAs (Si-Piwil2#1 and Si-Piwil2#2) or control siRNA (Si-control) or Oligofectamine alone (mock) for 48 h, following which cells were harvested for analysis of Piwil2 expression by Western blot. (b) Growth curves were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each point indicates the mean of spectrometric absorbance ± SD of three independent experiments (*$P < 0.001$). (c) Quantification of colony formation is presented as mean values ± SD relative to control transfectants of three independent experiments (*$P < 0.001$). (d) Protein-levels of caspase-3, poly(ADP-ribose) polymerase (PARP) and Bcl-2 were analyzed in treated cells by Western blot. (e) Mouse weight and tumor volume (f) (showed as indicated days) were determined in Piwil2 siRNA-treated cells and control siRNA-treated cells. (*$P < 0.001$). (A color version of this figure is available in the online journal)

expression of Piwil2 compared with Si-Piwil2#2, whereas no substantial effect on Piwil2 expression was found in the control siRNA-transfected groups (Figure 2a). Based on this result, we used Si-Piwil2#1 to knockdown Piwil2 expression for assessing the role of Piwil 2 in colon cancer cell proliferation *in vitro*. Using MTT assays, we showed no significant difference in the growth rate of mock-transfected and control-siRNA-transfected cells over a 96-h period. However, the growth of Piwil2-siRNA cells was significantly slower compared with mock or control-siRNA-transfected cells starting at 24 h after transfection ($P < 0.001$; see Figure 2b). Using colony formation assays in soft agar, we also showed a dramatic reduction in the number of colonies in Piwil2-siRNA cells compared with the two control groups ($P < 0.001$; see Figure 2c).

We investigated the role of Piwil2 in proliferation of colon cancer cells by evaluating apoptosis and death receptor signal pathways in Piwil2 knockdown cells. We tested the protein expression levels of activated caspase-3 and PARP, two downstream effectors molecules of the death receptor pathway. Western blot analysis revealed that the expression of activated caspase-3 and PARP was significantly

upregulated in the Piwil2 knockdown cells (Figure 2d). The mitochondrial apoptotic pathway can be negatively modulated by the antiapoptotic Bcl-2 family members, which play an important role in apoptotic control leading to cytochrome *c* release from mitochondria. We showed that Piwil2 knockdown resulted in a reduction of Bcl-2 protein levels. These findings suggest that Piwil2 might serve as a critical regulator for proliferation via the apoptotic pathway. Based on the *in vitro* findings described above, we investigated whether Piwil2 silencing was effective in suppressing tumor growth *in vivo*. Mice injected with Piwil2-siRNA cells showed delayed tumor onset and reduced tumor growth compared with mice injected with control shRNA-transfected cells (Figure 2e and f).

Gene silencing of Piwil2 suppressed migratory and invasive ability of colon cancer cells through modulation of MMP9 transcriptional activity

As Piwil2 was linked to advanced clinical stage and poorer metastasis-free survival of colon cancer patients, we investigated the role of Piwil2 on cell migration and invasion activity *in vitro*. We used migration chambers to evaluate the effect of Piwil2 knockdown on migration potency of

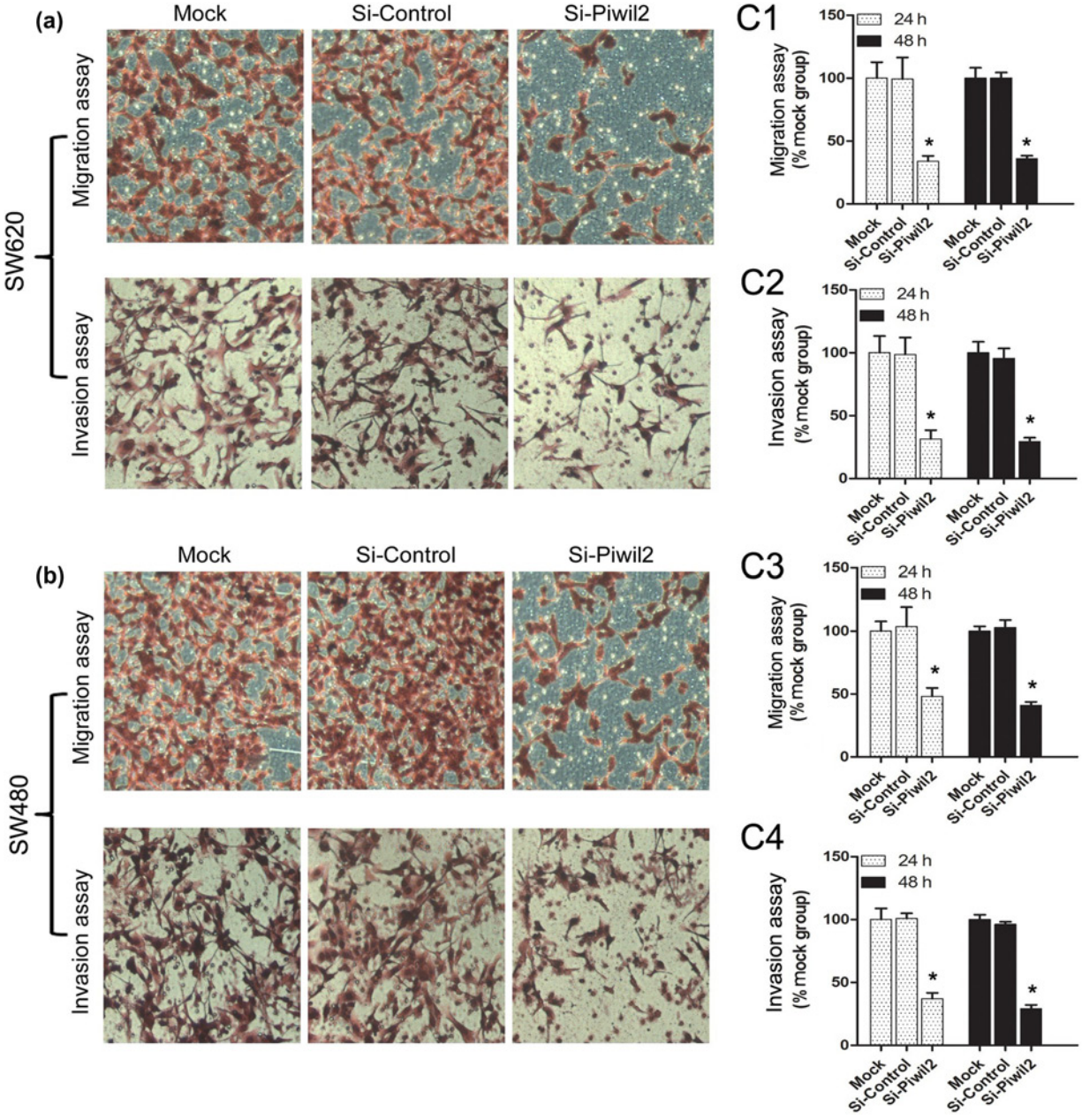

Figure 3 Piwil2 inhibition suppressed the migration and invasion of colon cancer cells. (a) Representative images with summarized quantitative data show migration and invasion of SW620 cells through transwells measured by direct counting of trespassed cells (×200). (b) Representative images with summarized quantitative data show migration and invasion of SW480 cell through transwells measured by direct counting of trespassed cells (×200). (c) and (d) The quantitative data are presented as mean relative numbers of migrated cells from four fields. (*$P < 0.001$, versus mock and Si-Control group). All experiments were performed in triplicate. (A color version of this figure is available in the online journal)

two colon cancer cell lines, SW620 and SW480. We confirmed efficient knockdown of Piwil2 expression in SW620 and SW480 cell lines using the Si-Piwil2#1 sequence (data not shown). Compared with the control groups, the Piwil2 siRNA group exhibited significantly reduced migratory ability in SW620 as well as SW480 cells at 24 and 48 h (Figure 3a, b, C1 and C3, $P < 0.001$). We also assessed the effect of Piwil2 depletion on tumor invasion. Our data showed that disruption of endogenous Piwil2 expression attenuated cell invasive potential in SW620 and SW480 colon cancer cells at 24 and 48 h (Figure 3a, b, C2 and C4, $P < 0.001$). To better understand the mechanism of Piwil2 in the progression and metastasis of colon cancer cells, we explored its effect on possible downstream metastasis-related molecules. MMP9 was previously shown to play a critical role in mediating tumor microenvironment, leading to enhanced cancer cell motility, angiogenesis and cancer growth.[19] However, the mechanism driving aberrant MMP9 activity in colon cancer remains unclear. Our data showed that knockdown of endogenous Piwil2 led to substantial reduction in both mRNA and protein levels of MMP9 (Figure 4a and b). Zymography assays showed that Piwil2 silencing also attenuated the enzyme activity of MMP9 (Figure 4c). We generated an MMP9 promoter-driven luciferase reporter vector PGL3-MMP9-2.0 containing a 2 kb MMP9 promoter sequence and used the dual luciferase assay to show that Piwil2 knockdown inhibited the transcriptional activity of MMP9 (Figure 4d). The data suggest a potential therapeutic role of Piwil2 in colon cancer, which may partially function via mediating downstream MMP9 activity.

Discussion

Although Piwil genes are considered to play important roles in RNA interference, stem cell self-renewal and translational regulation in germ cells,[20–25] relatively little is known about their roles in human disease, especially in malignances. In this study, we investigated the role of Piwil2 in the carcinogenesis and progression of colon cancer. We demonstrated that (a) aberrant upregulation of Piwil2 was significantly associated with more aggressive tumors, advanced clinical stage, LNM and advanced tumor stage in colon cancer; (b) the Piwil2 gene could be used as a novel prognostic marker for the progression and metastasis of colon carcinoma patients; (c) Piwil2 inhibition results in decreased proliferation and increased apoptosis of colon cancer cells; and (d) Piwil2 promotes the migration and invasion of colon cancer cells through activation of MMP9.

HIWI, a human homologue of PIWI, was recently shown to be highly expressed in colorectal cancer tissue and was shown to be a prognostic factor for LNM.[26] Silencing the HIWI gene was also shown to result in G2/M cell cycle arrest and growth inhibition in gastric cancer cells.[12] The

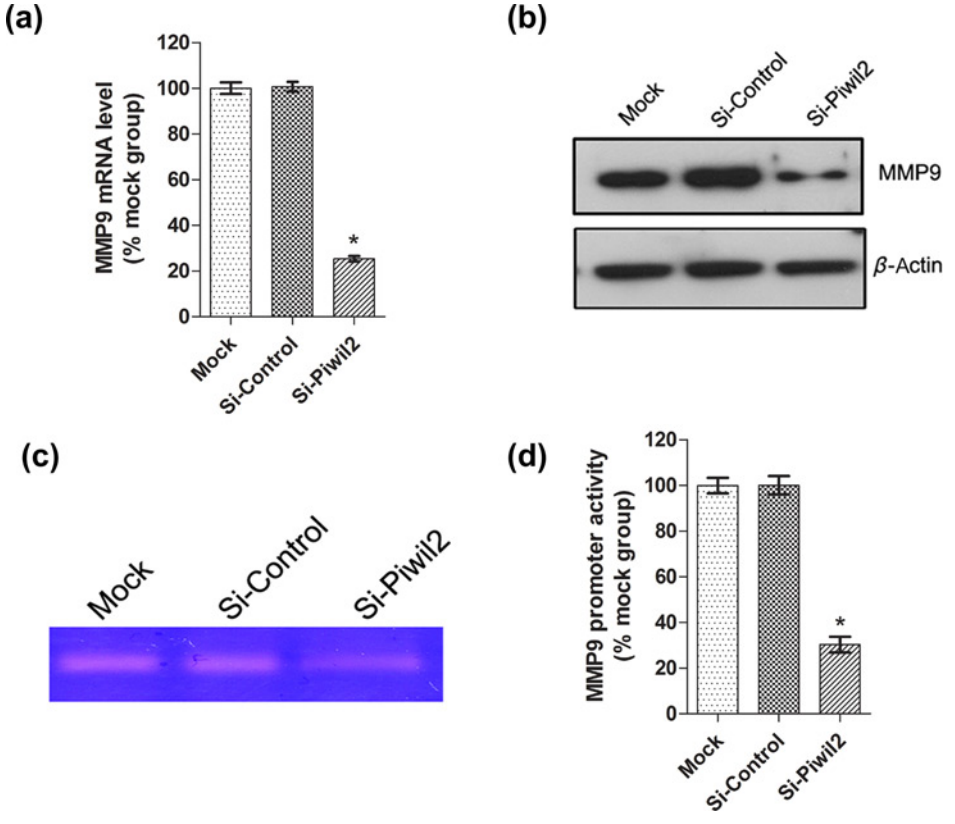

Figure 4 Piwil2 silencing depleted the activation of matrix metallopeptidase 9 (MMP9). Realtime polymerase chain reaction (a), Western blot (b) and zymography assay (c) were performed to examine the expression level and enzyme activity of MMP9 in the siRNA and control groups. (d) Dual-luciferase assay was performed to detect the promoter activity of MMP9 in the treated groups. Piwil2 knockdown reduced the transcriptional activity of MMP9 (*$P < 0.001$). (A color version of this figure is available in the online journal)

Piwil2 gene was recently shown to be overexpressed in a number of cancers.[15,10,14] However, since Piwil2 expression in colon cancer has not been extensively studied, we compared Piwil2 expression in primary colon cancer tissue and paired adjacent normal colonic mucosa and showed a significant elevation of Piwil2 expression in cancerous tissue compared with normal colonic mucosa. Piwil2 was previously suggested to play a role in the early stages of tumorigenesis in both breast cancer and seminomas, but not in normal tissues.[15,27] High Piwil2 expression was also seen in various stages of human cervical squamous cell carcinomas and adenocarcinomas. Additionally, Piwil2 was detected in some metaplastic epithelial cells adjacent to malignant lesions in cervical cancer.[14] Our data were consistent with these studies and suggested a potential role of Piwil2 in the onset of carcinogenesis in colon cancer.

It is thought that dysregulation of certain genes in tumor stem cells may mediate the pathological stages of atypical hyperplasia, dysplasia and carcinoma *in situ* and invasive/metastatic carcinoma.[9,28] P2L2 (Piwil2-like) proteins were previously shown to promote tumor cell survival and proliferation via upregulation of Bcl-2 and Stat3, and were always co-expressed with NFκB.[25] Piwil2 was also shown to directly interact with Stat3 and bind the p53 promoter to suppress p53 activity.[13] In order to further elucidate the mechanism underlying the role of Piwil2 in tumorigenesis and metastasis, we evaluated Piwil2 levels in metastatis and paired primary colon tumor lesions. Our data showed a significant upregulation of Piwil2 in LNM tissue when compared with paired primary colon tumor lesions, suggesting that Piwil2 is associated with tumor metastasis. Since motility and invasion play a critical role during the process of metastasis,[29] we investigated the underlying mechanism of Piwil2 in the metastasis of colon cancer using a migration and invasion assay. We showed that Piwil2 silencing significantly suppressed the migratory and invasive ability of colon cancer cells. We also found that Piwil2-positive patients had a nearly three-fold higher risk (HR 2.63) of metastasis after colectomy relative to Piwil2-negative patients. Our findings provided the first evidence that aberrant overexpression of Piwil2 could serve as a prognostic marker for metastasis in colon cancer patients.

We asked if Piwil2 could serve as a novel anticancer target in colon cancer. Silencing Piwil2 was previously shown to result in downregulation of the Stat3/Bcl-XL pathway and induction of apoptosis in breast cancer cells.[30] These results agreed with our data, which showed that Piwil2 silencing inhibited proliferation and induced apoptotic cell death via modulation of Bcl-2, caspase-3 and PARP levels in colon cancer cells. Our data provided the first evidence that Piwil2 inhibition can trigger both the death receptor pathway and the mitochondrial apoptotic pathway in colon cancer, and suggest that Piwil2 might be used as a novel molecular target for therapy in colon cancer patients. Importantly, blockade of Piwil2 function was also sufficient to slow down tumor growth of SW620 cancer cells in mice, suggesting a critical role of Piwil2 for survival and/or growth of cancer cells.

The mechanism underlying upregulation of Piwil2 expression in the aggressive colon cancer phenotypes remains unclear. There is emerging evidence to show that Piwil2 phosphorylates and enhances the DNA-binding activity of signal transducer and activator of transcription 3 (STAT3) to the p53 promoter, resulting in silencing of the p53 gene in cancer cells.[13] Piwil2 was also recently shown to independently target the Stat3/Bcl-X and Stat3/cyclin D1 in testicular germ cell tumors, and enforced expression of Piwil2 in mouse fibroblasts reduced apoptosis, increased colony formation and induced transformation.[10] In this study, we showed that Piwil2 silencing triggered a decrease in MMP9 activation. It will be interesting to investigate if Piwil2 could act as an oncogenic factor in tumorigenesis of various tissues via multiple signaling molecules.

In conclusion, to our best of knowledge, this is the first study showing the expression of Piwil2 in colon cancer tissues, and demonstrating the clinical significance of Piwil2 in colon cancer. High Piwil2 expression could be a significant prognostic marker of poor survival in colon cancer patients. Piwil2 may also serve as a useful indicator for tumor progression and metastasis which could be partially mediated via modulation of MMP9 activation. We are presently not aware of any reports showing a link between Piwil2 and any other metastasis-related markers. Our study provides a basis for further investigation of Piwil2 as a prognostic and therapeutic marker in colon cancer.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. DL, XS, DY and QL conducted the experiments; HT supplied critical reagents for immunohistochemistry and *in vitro* experiments; and DL wrote the manuscript. DL, XS and DY contributed equally to this work.

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