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Inhibition of PRRX2 suppressed colon cancer liver metastasis via inactivation of Wnt/ β -catenin signaling pathway

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ABSTRACT

The aim of this study was to investigate whether PRRX2 may regulate the liver metastasis of colon cancer via the Wnt/ β -catenin signaling pathway. PRRX2 and β -catenin in patients with the liver metastases of colon cancer were detected by immunohistochemistry. Colon cancer cells (CT-26 and CMT93) were divided into Normal, si-Ctrl, si-PRRX2 and si-PRRX2 + LiCl groups. Cell invasive and migrating abilities and the related proteins were detected. Liver-metastatic mice model was constructed consisting of Normal, NC shRNA and PRRX2 shRNA groups to examine the function of PRRX2 shRNA on liver metastasis. We found that PRRX2 and β -catenin positive rate was elevated in colon cancer tissues, especially in those tissues with liver metastasis, and there was a close relation between PRRX2 and the clinical staging, lymph node metastasis and numbers of liver metastases of colon cancer patients with liver metastasis. In vitro, the invasive and migrating abilities of CT-26 and CMT93 cells decreased apparently in the si-PRRX2 group, with down-regulation of PRRX2, p-GSK3 β ^{ser9}/GSK3 β , nucleus and cytoplasm β -catenin, TCF4 and Vimentin but up-regulation of E-cadherin. However, LiCl, the Wnt/ β -catenin pathway activator, can reverse the inhibitory effect of si-PRRX2 on invasive and migrating ability of colon cancer cells. In vivo, the volume and weight of transplanted tumor and the number of liver metastases in the PRRX2 shRNA group were significantly reduced, with the similar protein expression patterns as in vitro. In a word, PRRX2 inhibition may reduce invasive and migrating abilities to hinder epithelial-mesenchymal transition (EMT), and suppress colon cancer liver metastasis through inactivation of Wnt/ β -catenin pathway.

1. Introduction

Colorectal cancer is one of the most common malignant tumors of digestive tract, ranking second in female and third in male regarding its incidence [4]. Currently, the morbidity and mortality of colon cancer keep increasing year by year and its primary therapy is surgery, but its therapeutic effect is unsatisfactory for those advanced or metastasis patients [18]. It was previously shown that tumor metastasis remained to be the major cause of death and treatment failure in most cancer patients [15], while the metastasis occurred through a hematogenous route to the liver was credited as the primary cause of death of colon cancer patients [5]. At the meantime, the prognosis of colon cancer is closely related to metastasis, especially liver metastasis, thus inhibiting liver metastasis is likely to improve its therapeutic efficacy [21,33]. Therefore, looking for a specific and effective metastasis-related gene is

urgently needed for the treatment of colon cancer liver metastasis.

Paired-related homeobox transcription factors (PRRX) was identified to participate in the cell differentiation and the development of organ tissues [12], and had three highly-conserved genes, including PRRX1, PRRX2 and PRRX3, among which PRRX1 and PRRX2 had great similarity of 97% in homologous domain [13,25]. PRRX1 and PRRX2 are important factors for the development of mesenchymal tissues and involved in the organogenesis of many tissues during developmental processes [13]. At present, there is compelling evidence pointing out the association of PRRX2 with some tumors, including prostate cancer [24], esophageal squamous cell carcinoma (ESCC) [41] and leukemia [8]. A recent study reported that PRRX2 as a TGF- β -induced factor that enhances invasion and migration in breast cancer [16]. Besides, PRRX2 expression has been found to be abnormally expressed in metastatic gastric cancer, which acted as a potential biomarker for gastric cancer

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metastasis [6]. In the study of Lv Z et al., silencing PRRX2 can inhibit the Wnt/ β -catenin signaling pathway to hinder the epithelial-mesenchymal transition (EMT) process of breast cancer cells, thereby inhibiting tumor invasion and migration [19]. As is known to all, the classical Wnt/ β -catenin pathway is essential for the involvement of various pathological processes in many types of tumors (including colon cancer), such as promoting tumor cell proliferation and inhibiting tumor cell apoptosis [30,42]. For instance, downregulation of CDK-8 in the work of Cai et al. could suppress the activity of Wnt/ β -catenin to inhibit the liver metastasis of colon cancer cells [2]. In light of this, it is reasonable for us to hypothesize that PRRX2 may also play a great role in colon cancer metastasis by mediating the Wnt/ β -catenin pathway.

Therefore, the current study was designed to investigate the effects of PRRX2 knockdown on the invasion and metastasis of colon cancer cells. Also, the liver metastasis model of colon cancer in nude mice was constructed to further investigate its effect *in vivo*.

2. Materials and methods

2.1. Ethics statement

Written informed consent was obtained from all participants before the study. The collection and use of all tissue samples abided by ethical principles and approved by the Ethical Committee of Clinical Experiments of Gansu Provincial People's Hospital. All animal experiments in this study gained the approval of the Ethics Committee of Laboratory Animals in our hospital and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH) [1].

2.2. Study subjects

From December 2013 to December 2017, 96 colon cancer patients who received surgery in our hospital were recruited in this study, among whom 40 cases had liver metastasis and the other 56 cases had no metastasis. None of patients had received any radiotherapy, chemotherapy or immuno-biological cancer therapy before surgery. At the same time, the normal colon tissues about 5–10 cm to the tumor margin, confirmed healthy by postoperative pathology, were collected from 56 patients as normal control group. All pathological diagnoses were confirmed by central pathologic review by two independent pathologists.

2.3. Immunohistochemical staining

Tissue samples were fixed in 4% formalin and dehydrated with gradient alcohol. Then the samples were embedded in paraffin (Thermo), sliced into serial sections (5 μ m in thickness), and de-waxed, followed by antigen retrieval and blocking. Next, one drop of primary antibody PRRX2 and β -catenin (Sigma-Aldrich, 1:500 diluted) was added onto the section for overnight incubation at 4 °C. On the next day, sections were washed with PBST, after which the biotinylated secondary antibody (1:500 diluted, Abcam) was added for incubation (2 h) at 37 °C. Later, ABC compound was added for 1 h of incubation at 37 °C, before development with DAB, section mounting, observation and picture-taking under an optical microscope. Scoring was conducted according to the ratio and intensity of positive-staining cells: 0–5% scored 0; 6–35% scored 1; 36–70% scored 2; more than 70% scored 3. The final score of PRRX2/ β -catenin expression was classified as negative (score 0–1) or positive (score 2–3) [20].

2.4. Cell culture, transfection and grouping

Colon cancer CT-26 and CMT93 cell lines were provided by Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). The cells were grown in RPMI 1640 complete culture medium containing

10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin in an incubator with 5% CO₂ and 90% saturated humidity. After cell confluence reached 80%–90%, the appropriate amount (0.25%) trypsin was added for digestion and passaging. One day before transfection, cells were spread onto the culture plate. PRRX2 siRNA and corresponding si-Ctrl were transfected into colon cancer cells respectively using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Then, Normal group (cells without transfection), si-Ctrl (cells transfected with siRNA negative control plasmids), si-PRRX2 group (cells transfected with PRRX2 siRNA), and si-PRRX2 + LiCl group (cells cultured in medium with 20 μ M Wnt/ β -catenin pathway activator LiCl (Sigma) before transfecting with PRRX2 siRNA) were divided. Both PRRX2 siRNA and siRNA negative control plasmids were synthesized by Shanghai GenePharma Co., Ltd.

2.5. Transwell assay

The diluted Matrigel (1 mg/ml, 40 μ l) was added into Transwell chamber for 1–2 h of incubation at 37 °C for solidification. After cells were transfected for 24 h, the serum-free RPMI culture medium was used to make single-cell suspension (4×10^5 cells/ml), and 200 μ l of which was added onto the Matrigel in the upper chamber. Next, 600 μ l culture medium containing 20% FBS was added to the bottom chamber as the chemokine. After 16 h of incubation at 37 °C with 5% CO₂, the chambers were taken out, cells were fixed in absolute methanol for 15 min at room temperature, and stained with 0.5% crystal violet 15 min. After drying in the air at room temperature, the membrane formed was removed and neutral resin was used for sealing. Cells were observed under an optical microscope.

2.6. Wound-healing assay

Briefly, cells were seeded into 24-well plates and every three wells were made as a set of replicates. When single layer of cells covered about 95% of the plate bottom, the scratch line was drawn along the middle of each well. The tip of 100 μ l pipetting gun was used to scratch a line vertical to the marking line across the right middle of each well. PBS was used to wash away cells not adherent to the bottom of the plate, by 3–5 times. Next, culture medium containing 0.5% FBS was added for the culture of cells. Photographs of cells were taken under a microscope. After measuring the width of scratch line at the crossing point of marking line and scratch line (0 h), cells were placed in an incubator for 48 h before the measurement and picture-taking of the new width of scratch lines. The difference of scratch line width was regarded as migrating distance for later statistical comparison.

2.7. Establishment of liver metastasis model of colon cancer in nude mice

Thirty Balb/c male nude mice (4-week-old, weighing 17–22 g) were purchased from Shanghai SLAC Laboratory Animal Co., LTD. Lentivirus plasmid containing short hairpin RNA of PRRX2 and negative control were designed by Genechem (Shanghai, China). These viruses were used to transduce CT-26 cells with of 5×10^5 cell/well in the presence of 8 μ g/ml polybrene in 6-well plates. Transduced cells were selected in DMEM containing 2 μ g/ml puromycin for 1 week. Thirty nude mice were evenly divided into three groups: Normal group, NC shRNA group, and PRRX2 shRNA group. Liver metastasis models of colon cancer were established in nude mice based on protocols written in previous studies [17,31]. For starters, nude mice were anaesthetized with 1% pentobarbital sodium (35 mg/kg) by intraperitoneal injection. An incision of 1 cm was cut under the junction of left axillary midline and rib margin, the spleen was found and taken out of the abdomen, and a micro-syringe was used to inject 0.2 ml CT-26 cell suspension into the capsule of spleen in mice from Normal group, NC shRNA group and PRRX2 shRNA group, which was put back into the abdomen. The status, food in-take and weight change of nude mice were observed every day. After 14

days, mice were killed, and the liver and spleen were taken out to investigate the degree of liver metastasis. The number of liver metastases on the surface of liver was counted for mice of each group. Tumor tissues were fixed with formaldehyde, embedded with paraffin, and sliced into sections for HE staining.

2.8. Western blotting

Total protein samples were isolated from tissues or cultured cells using RIPA lysis buffer (Beyotime, Shanghai, China) and quantified with BCA protein assay (Wuhan Boster Biological Technology.,LTD). A nuclear and cytoplasmic protein extraction kit (Thermo) was used to separate nuclear and cytoplasmic proteins following the manufacturer's instructions. Loading buffer was added into proteins for 10 min of heating at 95 °C and 30 µg of sample was loaded. Electrophoresis with 10% polyacrylamide gel (Wuhan Boster Biological Technology., LTD) was used to separate proteins with the voltage from 80 V to 120 V, and proteins were transferred to the PVDF membrane with the voltage of 100 mV for 70 min. Next, proteins were blocked in 5% BSA for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 °C, which including PRRX2 (ab22600, Abcam, USA), p-GSK3β^{Ser9} (ab131097, Abcam, USA), β-catenin (9587 s, CST), TCF4 (ab185736, Abcam, USA), E-cadherin (ab1416, Abcam, USA), Vimentin (ab137321, Abcam, USA) and β-actin (ab8226, Abcam, USA), all diluted by 1:1000. The membranes were then washed with TBST for three times, 5 min each time, and secondary antibody was added for 1 h of incubation at room temperature. After that, the proteins were perceived by the enhanced chemiluminescence kit (Invitrogen). With β-actin as the loading control, the gray value of target band was analyzed with the software Image J. This experiment was conducted independently for three times.

2.9. Statistical methods

Statistical software SPSS 21.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the statistical data in this study. Measurement data were presented by mean ± standard deviation, and comparison among multiple groups was performed by using One-Way ANOVA, while between two groups analyzed thorough Post-hoc Tukey's test. Enumeration data were expressed as percentage or ratio and tested by using Chi-square test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PRRX2 expression in colon cancer tissues

The positive rate of PRRX2 and β-catenin expression detected by immunohistochemical staining turned out to be higher in colon cancer tissues than normal tissues, especially in the colon cancer tissues with liver metastases (all $P < 0.05$, Fig. 1). After analyzing the clinicopathological characteristics of patients, we found that PRRX2 expression in colon cancer patients and patients with liver metastases had no correlation to the gender, age, and tumor differentiation degree of patients (all $P > 0.05$), but it was closely associated with the clinical staging, lymph node metastasis and numbers of liver metastases of patients (both $P < 0.05$, Table 1). The results in Table 1 also showed that PRRX2 expression positively correlated with the expression of β-catenin (all $P < 0.05$).

3.2. Effect of PRRX2 on the invasion and migration of colon cancer cells

As detected by Transwell and wound-healing assays shown in Fig. 2, when compared to the Normal group, the number of invasive and migrating cells were obviously decreased in the CT-26 and CMT93 cells from the si-PRRX2 group (all $P < 0.05$), but were not differ from those

in the si-Ctrl group (all $P > 0.05$). Besides, the invasive and migrating cell numbers were higher in the si-PRRX2 + LiCl group than in the si-PRRX2 group (all $P < 0.05$).

3.3. Expression of Wnt/β-catenin pathway-related proteins in colon cancer cells

Western blotting was used to detect the expression of Wnt/β-catenin pathway-related proteins (Fig. 3). Compared with Normal group, CT-26 and CMT93 cells in the si-Ctrl group had no obvious difference in pathway-related proteins (all $P > 0.05$), although si-PRRX2 group presented significant decreases in the expression levels of PRRX2, p-GSK3β^{Ser9}/GSK3β, nucleus and cytoplasm β-catenin, TCF4 and Vimentin, as well as an increase in E-cadherin (all $P < 0.05$). Besides, the colon cancer cells in the si-PRRX2 + LiCl group exhibited higher protein expressions of PRRX2, p-GSK3β^{Ser9}/GSK3β, nucleus and cytoplasm β-catenin, TCF4 and Vimentin but lower E-cadherin than those in the si-PRRX2 group (all $P < 0.05$).

3.4. Comparison of the volume and weight of transplanted tumors in nude mice

No mouse died during the experiment and transplanted tumor can be found in the spleen of mice in all groups. HE staining was used to confirm histologically the formation of splenic tumor in situ (Fig. 4A), and liver tissues was also used to perform HE staining, which showed the formation of liver metastases (Fig. 4B). As illustrated in Fig. 4C, D, nude mice in the Normal group and NC shRNA group had enlarged tumor volume, and there was no significant difference in tumor volume and weight between these two groups ($P > 0.05$). However, PRRX2 shRNA group was statistically smaller in tumor volume and significantly lighter in tumor weight than Normal group (both $P < 0.05$).

3.5. Comparison of liver metastases of nude mice in each group

Liver metastases could be observed on the surface of liver in nude mice, but not in other organs. As displayed in Fig. 5, nude mice in Normal group and NC shRNA group had liver congestion with cancer nodules of different size and number dispersed on the surface, and some nodules fused together. Meanwhile, nude mice in the PRRX2 shRNA group had almost normal liver, occasionally with few and small nodules of metastatic tumor. Compared with Normal group, NC shRNA group was not significantly different concerning the number of liver metastases ($P > 0.05$), while PRRX2 shRNA group appreciably declined in the number of liver metastases ($P < 0.05$).

3.6. Expression of PRRX2 and Wnt/β-catenin pathway in nude mice

Western blotting was used to detect the protein expression of PRRX2 and Wnt/β-catenin pathway in spleen tissues of nude mice (Fig. 6A, B). Compared with Normal group, NC shRNA group had no observable difference in PRRX2 and Wnt/β-catenin pathway-related proteins (all $P > 0.05$); nevertheless, nude mice in the PRRX2 shRNA group were significantly reduced in the protein expression of PRRX2, p-GSK3β^{Ser9}/GSK3β, nucleus and cytoplasm β-catenin, TCF4 and Vimentin, but appreciably increased in the expression of E-cadherin protein (all $P < 0.05$). Besides, we examined the expression of β-catenin by immunohistochemistry (Fig. 6C). Decreased expression of β-catenin was observed in the nude mice in the PRRX2 shRNA group compared with Normal group.

4. Discussion

First of all, a close association between PRRX2 expression and liver metastasis of colon cancer was identified in the current study, since PRRX2 was found to be significantly up-regulated in colon cancer

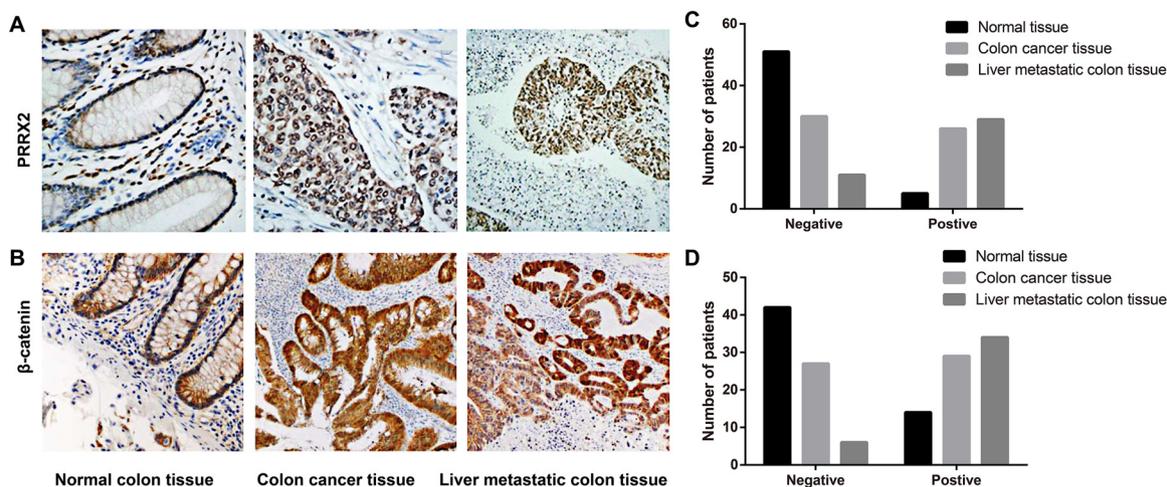


Fig. 1. PRRX2 and β -catenin expression in colon cancer tissues.

Note: A–B, Expression of PRRX2 (A) and β -catenin (B) in normal colon tissues and colon cancer tissues with/without liver metastases detected after immunohistochemical staining; C–D, The quantitative analysis of PRRX2 (C) and β -catenin (D) expression in normal colon tissues and colon cancer tissues with/without liver metastases.

tissues, especially higher in those tissues with liver metastases. Similar to our study, Yu-Lin Juang et al. revealed that PRRX2 was dramatically up-regulated in breast cancer tissues, and its high expression may lead to the promotion of TGF- β -induced breast tumor growth and partial epithelial mesenchymal transition (EMT) with a poorer prognosis [16]. Besides, the TGF- β 1-induced EMT in the papillary thyroid carcinoma cells were also accompanied with the up-regulation of PRRX1 [9]. Then, we also discovered the significant correlation of PRRX2 expression to the clinical staging and lymph node metastasis of colon cancer patients with liver metastases. As indicated by Takahashi Y et al., the higher PRRX1 predicted the metastasis and poor prognosis of colorectal cancer, promoting the EMT of colorectal cancer cells [34]. To our knowledge, EMT, a critical event in the progression of tumor metastasis, is a fundamental process of epithelial cells acquiring migrating and invasive functions [22,32]. Importantly, both PRRX1 and PRRX2 were confirmed to act as a EMT inducer in promoting EMT of tumor and maintaining the sternness of tumor in previous research [27], as well as

transforming growth factor- β (TGF- β), which has been shown to have important functions in various stages of carcinogenesis [28]. Recombinant protein TGF- β can directly stimulate tumor epithelial cells to trigger EMT process, and thereby promoting infiltration and metastasis of tumor cells [40]. As a matter of fact, TGF- β 1 and EMT markers were significantly increased in colon cancer tissues and closely correlated to the tumor cell metastasis [29]. Because of this, we suggested that PRRX2 upregulation in colon cancer might also be induced by TGF- β 1 through promoting EMT, especially in colon cancer tissues with liver metastases.

Additionally, we selected colon cancer cells to conduct transfection experiments *in vitro* and found that inhibiting PRRX2 can significantly down-regulate the invasive and migrating abilities of colon cancer cells. In agreement with our findings, silencing PRRX2 can reduce the EMT process and inhibit the invasion and migration of breast cancer cells by inhibiting Wnt/ β -catenin pathway, with the down-regulation of β -catenin, cyclin-D1, vimentin and α -SMA but the up-regulation of E-

Table 1

Relationship between PRRX2 expression and clinicopathological characteristics of colon cancer patients.

Clinicopathological characteristics	Colon cancer patients			Patients with liver metastasis		
	Positive expression (n = 55)	Negative expression (n = 41)	P	PRRX2		P
				Positive expression (n = 29)	Negative expression (n = 11)	
Gender			0.541			0.723
Male	30(54.55%)	25(60.98%)		16 (55.17%)	7 (63.64%)	
Female	25(45.45%)	16(39.02%)		13 (44.83%)	4 (36.36%)	
Age	63.45 \pm 11.51	60.38 \pm 10.47	0.183	62.79 \pm 10.98	58.00 \pm 12.49	0.242
Differentiation degree			0.681			1
Well-differentiated	31(56.36%)	21(51.22%)		15 (51.72%)	5 (45.45%)	
Moderate/low-differentiated	24(43.64%)	20(48.78%)		14 (48.28%)	6 (54.55%)	
TNM staging						0.014
I-II	11(20.00%)	24(58.54%)	< 0.001	4 (13.79%)	6 (54.55%)	
III-IV	44(80.00%)	17(41.46)		25 (86.21%)	5 (45.45%)	
Lymph node metastasis			0.006			0.020
With	39(70.91%)	17(41.46%)		23 (79.31%)	4 (36.36%)	
Without	16(29.19%)	24(58.54%)		6 (20.69%)	7 (63.64%)	
Numbers of liver metastases						0.007
One				6 (20.69%)	8 (72.73%)	
Multiple				23 (79.31%)	3(27.23%)	
β -catenin expression			< 0.001			0.039
Negative (n = 33)	6(10.91%)	27(65.85%)		2(6.90%)	4(36.36%)	
Positive (n = 63)	49(89.09%)	14(34.15%)		27(93.10%)	7(63.64%)	

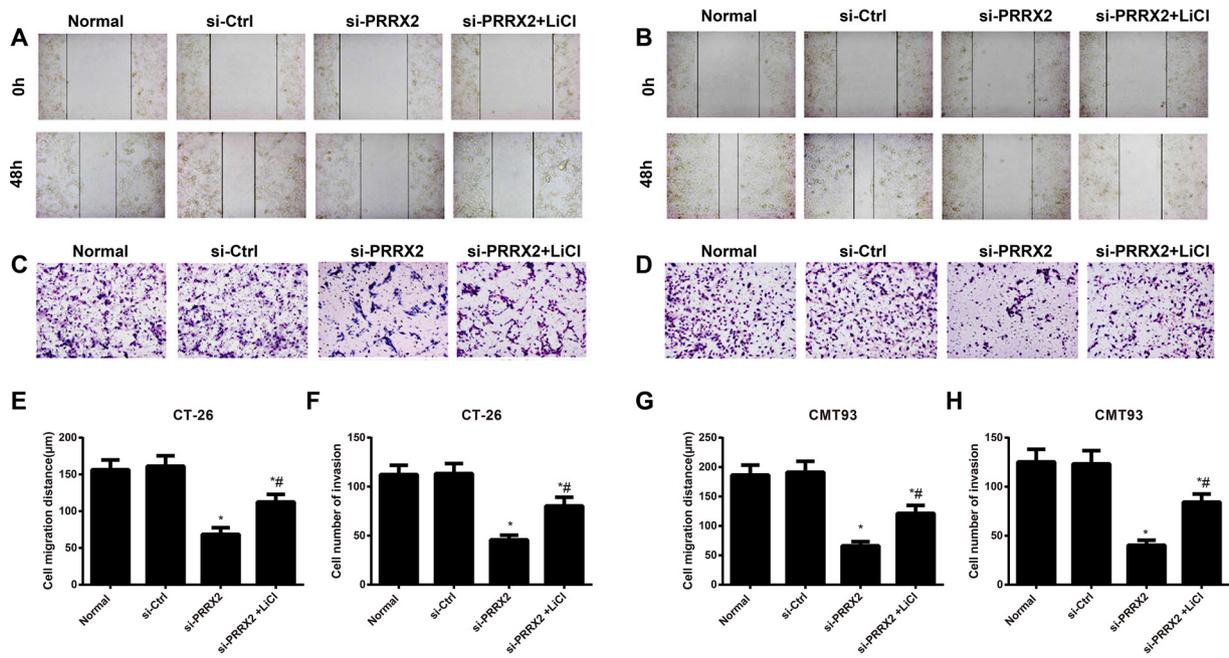


Fig. 2. The invasive and migrating abilities of colon cancer (CT-26 and CMT93) cells in each group. Note: A–B, The migrating ability of CT-26 (A) and CMT93 (B) cells detected by wound-healing assay; C–D, The invasion of CT-26 (C) and CMT93 (D) cells detected by Transwell assay; E–H, The quantitative analysis of the migration and invasion of CT-26 (E, F) and CMT93 (G, H) cells in each group; *, $P < 0.05$ compared with Normal group; #, $P < 0.05$ compared with si-PRRX2 group.

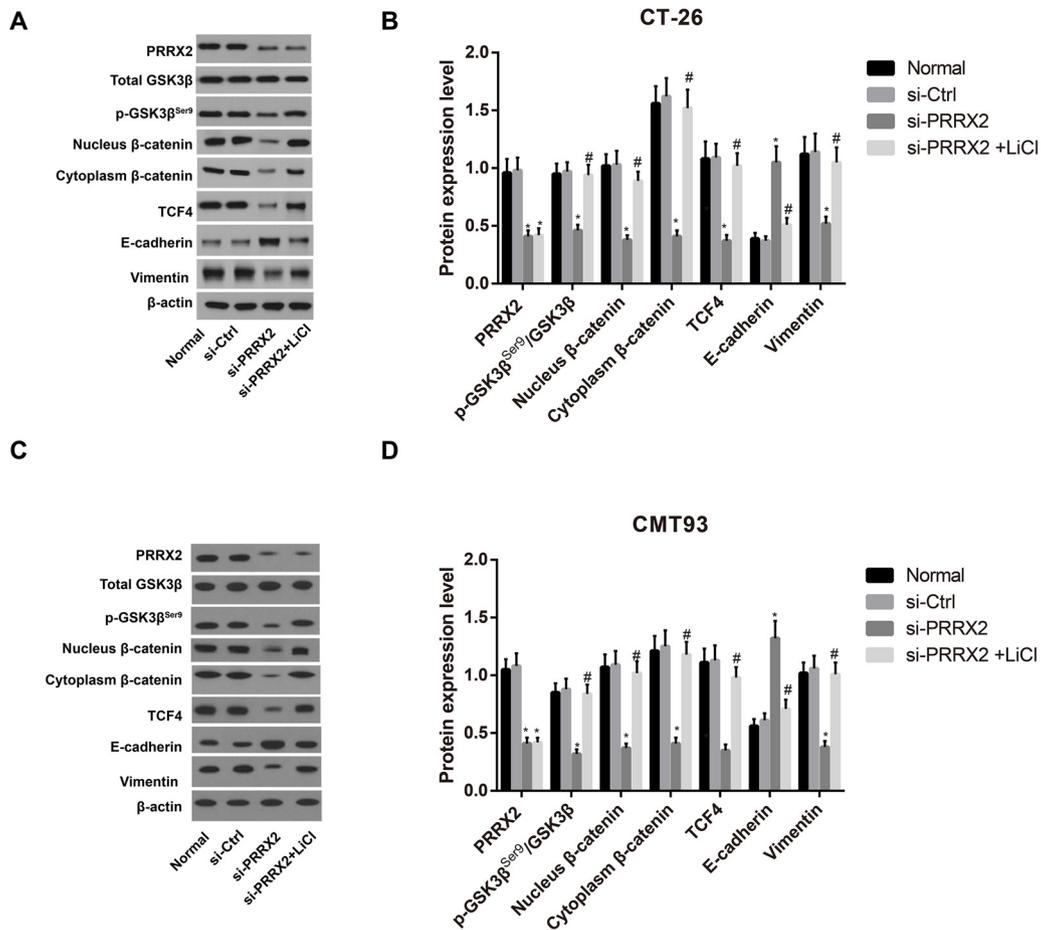


Fig. 3. Expression of Wnt/ β -catenin pathway-related proteins in colon cancer cells of each group. Note: A–D, Expression of Wnt/ β -catenin pathway-related proteins in colon cancer CT-26 (A, B) and CMT93 (C, D) cells detected by Western blotting; *, $P < 0.05$ compared with Normal group; #, $P < 0.05$ compared with si-PRRX2 group.

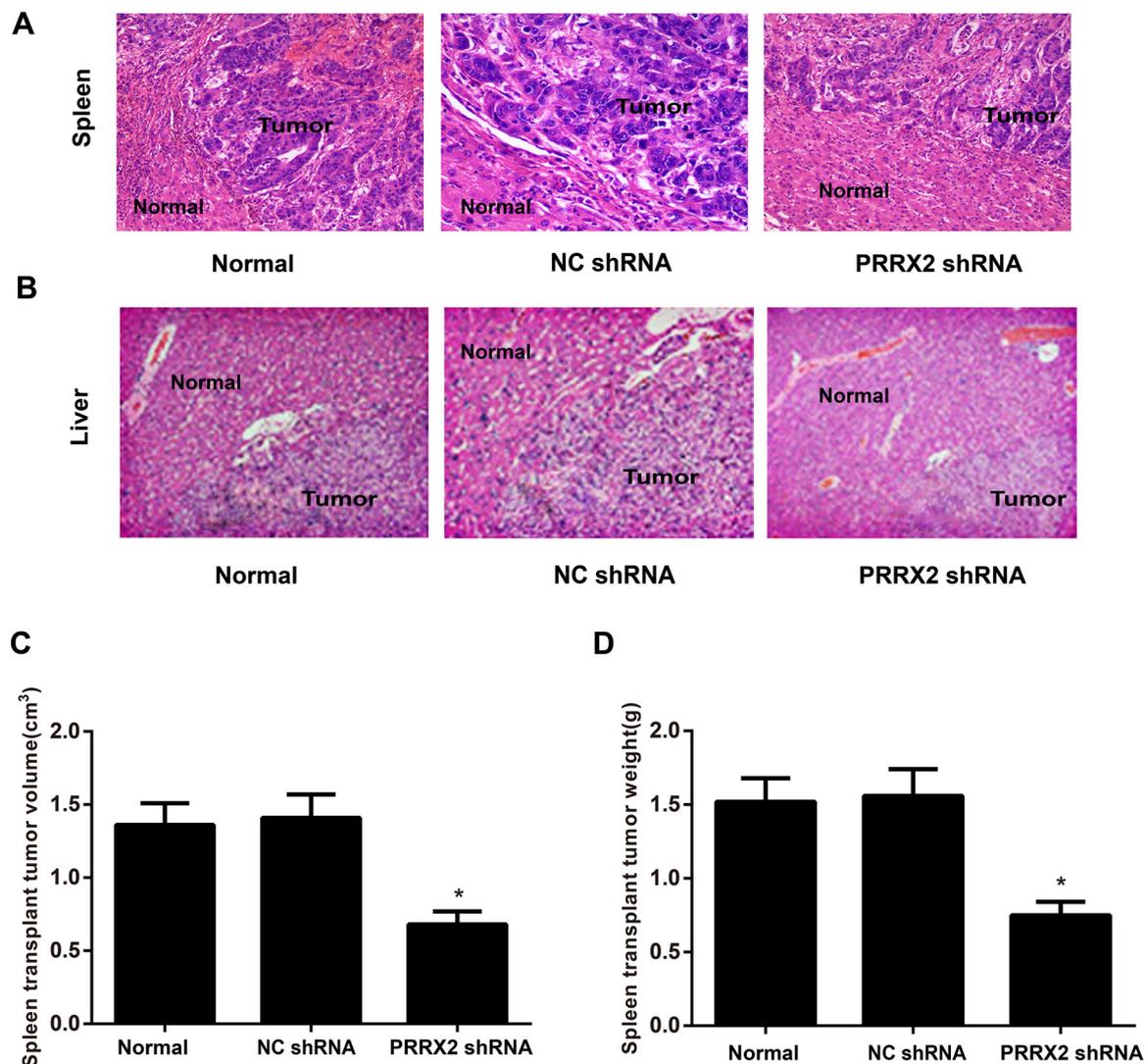


Fig. 4. Comparison of the volume and weight of transplanted tumors in nude mice of each group.

Note: A–B, HE staining analysis of spleen (A) and liver (B) tissues of nude mice in each group; C–D, The volume and weight of transplanted tumor in spleen of nude mice in each group; *, $P < 0.05$ compared with Normal group.

cadherin [19]. It has been well-known that E-cadherin is the key factor involved in EMT process and its down-expression is fundamental for the initiation of EMT and the promotion of tumor cell invasion and migration [7]. Vimentin is a structural protein distributed in mesenchymal cells and its expression level reflects the cell invasive and migrating abilities, which could act as a biomarker of malignant tumors [14]. Thus, we also performed a western blotting assay in our work to detect some related molecules, and consequently, inhibiting PRRX2 can down-regulate the protein expression of p-GSK3^{Ser9}, β -catenin, TCF4 and Vimentin, and up-regulate the expression of E-cadherin. However, Wnt/ β -catenin pathway activator LiCl can reverse the inhibitory effect of PRRX2 siRNA on colon cancer cell metastasis, indicating that PRRX2 inhibition exerted its effect via the regulation of Wnt/ β -catenin signaling pathway. In general, when Wnt signals are abnormally activated, Wnt protein would bind to FZ/LRP receptor, and Dvl in cytoplasm would phosphorylate GSK-3 β to dissociate Axin-ApC-GSK3 β , further blocking the phosphorylation of β -catenin, and resulting in the aggregation of free β -catenin in cytoplasm, which enters the nucleus to bind to LEF/TCF and specifically initiates and activates the downstream target genes in tumorigenesis and metastasis [23,26,36]. On the other hand, E-cadherin can form complex by binding to β -catenin in cytoplasm, and thereby reducing the amount of free β -catenin in cytoplasm

and inhibiting the formation of β -catenin/TCF complex, eventually counteracting the role of β -catenin/TCF complex in promoting tumor invasion and migration [10,11,38]. More notably, the activation of Wnt/ β -catenin signaling pathway breaks the E-cadherin/ β -catenin complex and triggers the EMT process, thereby promoting the invasion and metastasis of colon cancer [43]. These findings suggested that PRRX2 reduction may inhibit Wnt/ β -catenin pathway and hinder the EMT of colon cancer cells, and eventually blocking the invasion and migration of colon cancer cells *in vitro*. Moreover, we constructed the liver metastases model of colon cancer in nude mice to further verify our hypothesis *in vivo*. As a result, the nude mice transfected with si-PRRX2 decreased significantly in tumor volume, weight and numbers of liver metastases, with the restricted Wnt/ β -catenin pathway activity and EMT process in spleen tissues. Consistently, Lv Z et al. also found that PRRX2 inhibition can effectively suppress the growth of volume and weight of transplanted breast tumor and block the lung metastasis of breast cancer *in vivo* [19]. In a previous report by Takano S et al., PRRX1b promoted the invasion, differentiation and EMT of pancreatic cancer cells, while PRRX1a aided the liver metastasis and tumor differentiation of pancreatic cancer *in vivo* [35]. It is worth mentioning that EMT of tumor *in situ* weakens the ability of adhesion between cells and endows cells with the ability of local infiltration and distant

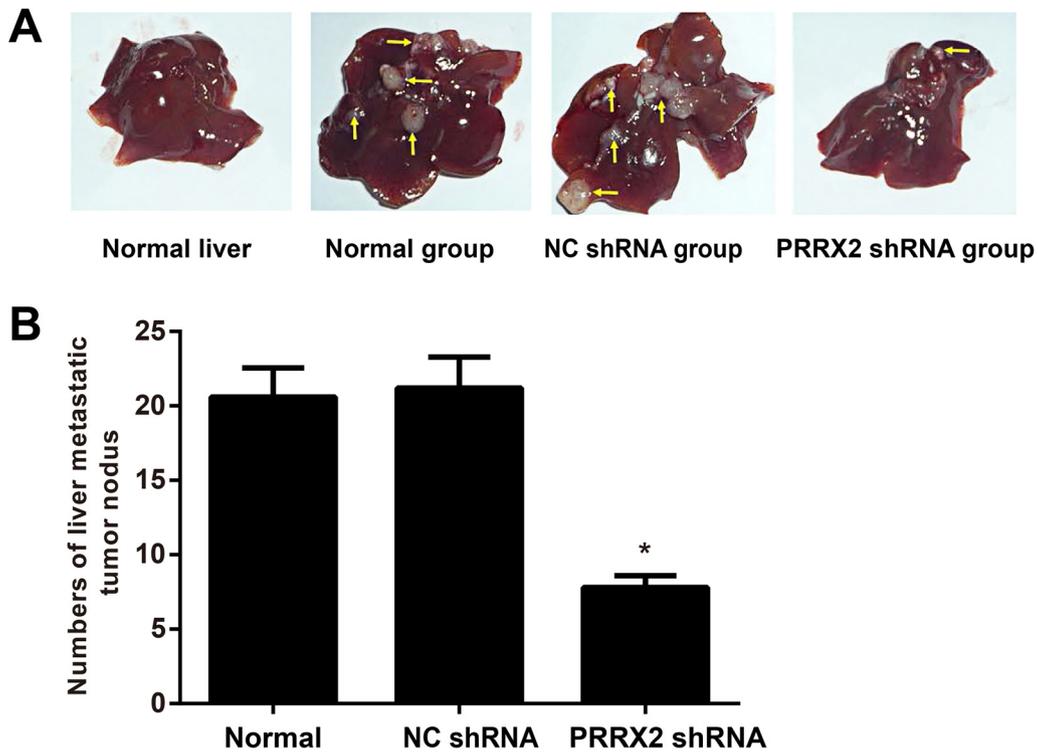


Fig. 5. Comparison of the liver metastases in nude mice of each group.

Note: A, The liver specimen of nude mice in each group; the metastatic tumor nodules are indicated with yellow arrows; B, The number of nodules of liver metastases in nude mice of each group; *, $P < 0.05$ compared with Normal group.

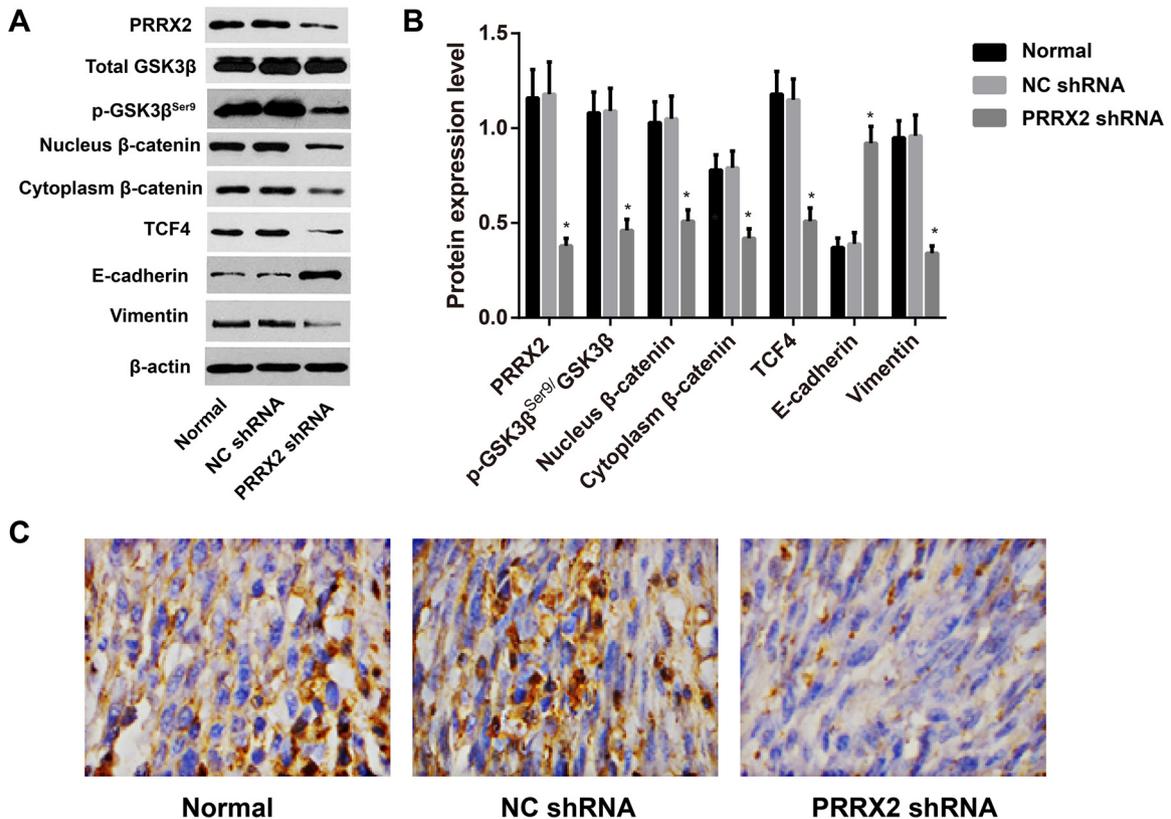


Fig. 6. Expression of PRRX2 and Wnt/β-catenin pathway-related proteins in nude mice of each group.

Note: A–B, Expression of PRRX2 and Wnt/β-catenin pathway-related proteins in nude mice of each group detected by Western blotting; C, Immunohistochemistry analysis of β-catenin expression in nude mice of each group; *, $P < 0.05$ compared with Normal group.

migration, so micro-metastasis is formed in target organs via the circulatory system and the metastasis of tumors *in vivo* is realized [3,37,39]. All these findings suggested that silencing PRRX2 may inhibit the tumor EMT *in situ* by down-regulating the Wnt/ β -catenin signaling pathway, suppressing the liver metastasis of colon cancer *in vivo*.

Based on these data, we indicated that inhibiting PRRX2 may suppress EMT process, reduce cell migration and invasion, and block the liver metastasis of colon cancer through down-regulation of Wnt/ β -catenin activity. Hence, this study provided a theoretical basis for the prevention and treatment of liver metastasis of colon cancer.

Declaration of Competing Interest

None.

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References

- Bayne, Revised guide for the care and use of laboratory animals available. American physiological society, *Physiologist* 39 (199) (1996) 208-111.
- W.S. Cai, F. Shen, Z. Feng, J.W. Chen, Q.C. Liu, E.M. Li, B. Xu, J. Cao, Downregulation of CDK-8 inhibits colon cancer hepatic metastasis by regulating Wnt/ β -catenin pathway, *Biomed. Pharmacother.* 74 (2015) 153-157.
- Y. Chao, Q. Wu, M. Acquafondata, R. Dhir, A. Wells, Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases, *Cancer Microenviron.* 5 (2012) 19-28.
- J. Deitrick, W.M. Pruitt, Wnt/ β catenin-mediated signaling commonly altered in colorectal Cancer, *Prog. Mol. Biol. Transl. Sci.* 144 (2016) 49-68.
- T. Du, H. Niu, Inhibitory effect of gene combination in a mouse model of colon cancer with liver metastasis, *Exp. Ther. Med.* 8 (2014) 913-918.
- D. Feng, X. Ye, Z. Zhu, Z. Wei, Q. Cai, Y. Wang, Comparative transcriptome analysis between metastatic and non-metastatic gastric cancer reveals potential biomarkers, *Mol. Med. Rep.* 11 (2015) 386-392.
- R.A. Foty, M.S. Steinberg, Cadherin-mediated cell-cell adhesion and tissue segregation in relation to malignancy, *Int. J. Dev. Biol.* 48 (2004) 397-409.
- C. Gervais, L. Mauvieux, N. Perrusson, C. Helias, S. Struski, V. Leymarie, B. Lioure, M. Lessard, A new translocation t(9;11)(q34;p15) fuses NUP98 to a novel homeobox partner gene, PRRX2, in a therapy-related acute myeloid leukemia, *Leukemia* 19 (2005) 145-148.
- H. Hardin, Z. Guo, W. Shan, C. Montemayor-Garcia, S. Asioli, X.M. Yu, A.D. Harrison, H. Chen, R.V. Lloyd, The roles of the epithelial-mesenchymal transition marker PRRX1 and miR-146b-5p in papillary thyroid carcinoma progression, *Am. J. Pathol.* 184 (2014) 2342-2354.
- M. Herzig, F. Savarese, M. Novatchkova, H. Semb, G. Christofori, Tumor progression induced by the loss of E-cadherin independent of β -catenin/Tcf-mediated Wnt signaling, *Oncogene* 26 (2007) 2290-2298.
- J. Heuberger, W. Birchmeier, Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling, *Cold Spring Harb. Perspect. Biol.* 2 (2010) a002915.
- M. Higuchi, T. Kato, M. Chen, H. Yako, S. Yoshida, N. Kanno, Y. Kato, Temporospatial gene expression of Prx1 and Prx2 is involved in morphogenesis of cranial placode-derived tissues through epithelio-mesenchymal interaction during rat embryogenesis, *Cell Tissue Res.* 353 (2013) 27-40.
- M. Higuchi, S. Yoshida, H. Ueharu, M. Chen, T. Kato, Y. Kato, PRRX1 and PRRX2 distinctively participate in pituitary organogenesis and a cell-supply system, *Cell Tissue Res.* 357 (2014) 323-335.
- J. Ivaska, Vimentin: Central hub in EMT induction? *Small GTPases* 2 (2011) 51-53.
- Y. Jing, Z. Han, S. Zhang, Y. Liu, L. Wei, Epithelial-Mesenchymal Transition in tumor microenvironment, *Cell Biosci.* 1 (2011) 29.
- Y.L. Juang, Y.M. Jeng, C.L. Chen, H.C. Lien, PRRX2 as a novel TGF- β -induced factor enhances invasion and migration in mammary epithelial cell and correlates with poor prognosis in breast cancer, *Mol. Carcinog.* 55 (2016) 2247-2259.
- T. Kitamura, T. Fujishita, P. Loetscher, L. Revesz, H. Hashida, S. Kizaka-Kondoh, M. Aoki, M.M. Taketo, Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model, *Proc Natl Acad Sci U S A* 107 (2010) 13063-13068.
- M.N. Kulaylat, J.F. Gibbs, Regional treatment of colorectal liver metastasis, *J. Surg. Oncol.* 101 (2010) 693-698.
- Z.D. Lv, H.B. Wang, X.P. Liu, L.Y. Jin, R.W. Shen, X.G. Wang, B. Kong, H.L. Qu, F.N. Li, Q.F. Yang, Silencing of Prx2 Inhibits the Invasion and Metastasis of Breast Cancer both In Vitro and In Vivo by Reversing Epithelial-Mesenchymal Transition, *Cell. Physiol. Biochem.* 42 (2017) 1847-1856.
- Z.D. Lv, Z.C. Yang, X.P. Liu, L.Y. Jin, Q. Dong, H.L. Qu, F.N. Li, B. Kong, J. Sun, J.J. Zhao, H.B. Wang, Silencing of Prx1b suppresses cellular proliferation, migration, invasion and epithelial-mesenchymal transition in triple-negative breast cancer, *J. Cell. Mol. Med.* 20 (2016) 1640-1650.
- H.Z. Malik, Z.Z. Hamady, R. Adair, R. Finch, A. Al-Mukhtar, G.J. Toogood, K.R. Prasad, J.P. Lodge, Prognostic influence of multiple hepatic metastases from colorectal cancer, *Eur. J. Surg. Oncol.* 33 (2007) 468-473.
- D.S. Micalizzi, S.M. Farabaugh, H.L. Ford, Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression, *J. Mammary Gland Biol. Neoplasia* 15 (2010) 117-134.
- T. Nakamura, F. Hamada, T. Ishidate, K. Anai, K. Kawahara, K. Toyoshima, T. Akiyama, Axin, an inhibitor of the Wnt signalling pathway, interacts with β -catenin, GSK-3 β and APC and reduces the β -catenin level, *Genes Cells* 3 (1998) 395-403.
- A.S. Nikitina, E.I. Sharova, S.A. Danilenko, T.B. Butusova, A.O. Vasiliev, A.V. Govorov, E.A. Prilepskaya, D.Y. Pushkar, E.S. Kostyukova, Novel RNA biomarkers of prostate cancer revealed by RNA-seq analysis of formalin-fixed samples obtained from Russian patients, *Oncotarget* 8 (2017) 32990-33001.
- R.A. Norris, K.K. Scott, C.S. Moore, G. Stetten, C.R. Brown, E.W. Jabs, E.A. Wulfsberg, J. Yu, M.J. Kern, Human PRRX1 and PRRX2 genes: cloning, expression, genomic localization, and exclusion as disease genes for Nager syndrome, *Mamm. Genome* 11 (2000) 1000-1005.
- R. Nusse, Wnt signaling, *Cold Spring Harb. Perspect. Biol.* 4 (2012).
- O.H. Oceana, R. Corcoles, A. Fabra, G. Moreno-Bueno, H. Aclouque, S. Vega, A. Barrallo-Gimeno, A. Cano, M.A. Nieto, Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prx1, *Cancer Cell* 22 (2012) 709-724.
- S. Petanidis, E. Kioseoglou, K. Domvri, P. Zarogoulidis, J.M. Carthy, D. Anastakis, A. Moustakas, A. Salifoglou, In vitro and ex vivo vanadium antitumor activity in (TGF- β)-induced EMT. Synergistic activity with carboplatin and correlation with tumor metastasis in cancer patients, *Int. J. Biochem. Cell Biol.* 74 (2016) 121-134.
- M.S. Pino, H. Kikuchi, M. Zeng, M.T. Herraiz, I. Sperduti, D. Berger, D.Y. Park, A.J. Iafrate, L.R. Zukerberg, D.C. Chung, Epithelial to mesenchymal transition is impaired in colon cancer cells with microsatellite instability, *Gastroenterology* 138 (2010) 1406-1417.
- A. Sebio, M. Kahn, H.J. Lenz, The potential of targeting Wnt/ β -catenin in colon cancer, *Expert Opin. Ther. Targets* 18 (2014) 611-615.
- S. Shimma, Y. Sugiura, T. Hayasaka, Y. Hoshikawa, T. Noda, M. Setou, MALDI-based imaging mass spectrometry revealed abnormal distribution of phospholipids in colon cancer liver metastasis, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 855 (2007) 98-103.
- J.P. Sleeman, J.P. Thiery, SnapShot: the epithelial-mesenchymal transition, *Cell* 145 (162) (2011) e161.
- A. Stein, H.J. Schmoll, Systemic treatment of liver metastases from colorectal cancer, *Ther. Adv. Med. Oncol.* 5 (2013) 193-203.
- Y. Takahashi, G. Sawada, J. Kurashige, R. Uchi, T. Matsumura, H. Ueo, Y. Takano, S. Akiyoshi, H. Eguchi, T. Sudo, K. Sugimachi, Y. Doki, M. Mori, K. Mimori, Paired related homeobox 1, a new EMT inducer, is involved in metastasis and poor prognosis in colorectal cancer, *Br. J. Cancer* 109 (2013) 307-311.
- S. Takano, M. Reichert, B. Bakir, K.K. Das, T. Nishida, M. Miyazaki, S. Heeg, M.A. Collins, B. Marchand, P.D. Hicks, A. Maitra, A.K. Rustgi, Prx1 isoform switching regulates pancreatic cancer invasion and metastatic colonization, *Genes Dev.* 30 (2016) 233-247.
- N. Takebe, P.J. Harris, R.Q. Warren, S.P. Ivy, Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways, *Nat. Rev. Clin. Oncol.* 8 (2011) 97-106.
- M. Tania, M.A. Khan, J. Fu, Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer, *Tumour Biol.* 35 (2014) 7335-7342.
- X. Tian, Z. Liu, B. Niu, J. Zhang, T.K. Tan, S.R. Lee, Y. Zhao, D.C. Harris, G. Zheng, E-cadherin/ β -catenin complex and the epithelial barrier, *J. Biomed. Biotechnol.* 2011 (2011) 567305.
- J.H. Tsai, J. Yang, Epithelial-mesenchymal plasticity in carcinoma metastasis, *Genes Dev.* 27 (2013) 2192-2206.
- H. Wang, H. Zhang, L. Tang, H. Chen, C. Wu, M. Zhao, Y. Yang, X. Chen, G. Liu, Resveratrol inhibits TGF- β 1-induced epithelial-to-mesenchymal transition and suppresses lung cancer invasion and metastasis, *Toxicology* 303 (2013) 139-146.
- B.L. Wu, L.W. Luo, C.Q. Li, J.J. Xie, Z.P. Du, J.Y. Wu, P.X. Zhang, L.Y. Xu, E.M. Li, Comprehensive bioinformatics analysis of the mRNA profile of fascin knockdown in esophageal squamous cell carcinoma, *Asian Pac. J. Cancer Prev.* 14 (2013) 7221-7227.
- H. Yao, E. Ashihara, T. Maekawa, Targeting the Wnt/ β -catenin signaling pathway in human cancers, *Expert Opin. Ther. Targets* 15 (2011) 873-887.
- B. Yue, C. Liu, H. Sun, M. Liu, C. Song, R. Cui, S. Qiu, M. Zhong, A positive feed-forward loop between LncRNA-CYTOR and Wnt/ β -Catenin signaling promotes metastasis of Colon Cancer, *Mol. Ther.* 26 (2018) 1287-1298.