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Original Article

TAFs contributes the function of PTPN2 in colorectal carcinogenesis through activating JAK/STAT signaling pathway

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Abstract: The morbidity and mortality of colorectal cancer (CRC) ranks fourth worldwide, moreover, the tumor microenvironment (TME) of CRC is quite complex, and is one of the necessary factors affecting promotion of tumor metastasis. PTPN2 is a tumor suppressor which plays an important role in cancer-related downstream molecular pathway. FSP-1 is highly-expressed in multiple types of tumor tissues and is a biomarker of stromal fibroblasts. To examine the function of PTPN2 in the metastasis of CRC, the study evaluated the co-expression level of PTPN2 and FSP-1 in CRC tissues by double staining, and demonstrated the relationship with clinical information about each patient. The roles of PTPN2 and FSP-1 were detected *in vitro* by proliferation and transwell assay through knockdown of expression level of PTPN2. Lower PTPN2 with higher FSP-1 expression was correlated with poor survival outcomes in CRC. TAFs contribute to the migration function of PTPN2 in CRC *in vitro* through inducing changes in the level of TGF-β1. Western blot and qRT-PCR assays were used to detect the mechanism of PTPN2 regulation of migration with TAFs in the JAK/STAT signaling pathway, moreover, TAFs contributed the function of PTPN2 in colorectal carcinogenesis *in vivo*. In summary, the study shed light on the effect of TAFs contributes the function of PTPN2 in colorectal carcinogenesis through activating JAK/STAT signaling pathway. In addition, double-staining assay could give us a unique perspective from which to study TME in CRC.

Keywords: CRC PTPN2 TAFs migration double staining

Introduction

Colorectal cancer (CRC) is one of the most common digestive tract malignant tumors, and its morbidity and mortality rank fourth in the world [1]. Every year, more than 1.2 million patients worldwide are diagnosed with CRC, and more than 600,000 people die from the disease. The regional distribution of CRC is quite different, among which the incidence of CRC is higher in developed areas. China is a low-incidence area of CRC, but the incidence is increasing year-on-year. Metastasis is one of the main causes of death in patients with CRC [2]. About 50% of CRC patients have distant metastasis after operation, and their five-year survival rate is less than 12%, therefore, a better understanding of the mechanisms that control CRC metastasis will provide oncologists with information on screening new biomarkers for patients at high risk of metastasis and provide more information for patient treatment, such as developing stronger treatment plans to reduce the incidence of distant metastasis.

PTPN2 is a non-receptor, protein tyrosine phosphatase, which plays an important role in tumourigenesis [3, 4]. PTPN2 was originally cloned from the cDNA library of T cells, and is therefore also known as T-cell phosphatase (T-cell Protein Tyrosine Phosphatase, TCPTP) [5]. It is located on chromosome 18. Although PTPN2 was originally cloned from T cells, subsequent studies indicated that it is expressed in many other cell types. The protein encoded by this gene has different isomers by different

splice, in which the 45 kD isomer is located in the nucleus and the 48 kD isomer is located in the endoplasmic reticulum. The difference in localization is caused by the amino acid sequence of the C-terminal non-catalytic region of PTPN2. The subcellular localization of PTPN2 is not invariable. Under certain conditions of stimulation, the protein also invokes a shuttle process between the nucleus and cytoplasm [6].

Fibroblast-specific protein 1 (FSP-1), also known as S100 calcium-binding protein A4 (S100A4), has a molecular weight of 10 kD, and is an important biomarker of stromal fibroblasts [7]. FSP-1 is highly expressed in a variety of tumor tissues, and the growth and invasion ability of tumor cells is related to the level of expression of FSP-1 protein [8]. As a calcium-binding protein, FSP-1 can bind to cytoskeleton protein, improve the movement of cancer cells, promote the migration of tumor mesenchymal microvascular smooth muscle, and participate in the growth of tumor cell abnormalities [9, 10], therefore, FSP-1 is a factor related to cell differentiation, tumor development, and metastasis, and can help to determine the prognosis.

To identify the function of PTPN2 in the metastasis of CRC, we assessed the level of expression of PTPN2 and FSP-1 in 562 CRC tissue samples by double-staining, and revealed the relationship with FSP-1 expression, patient clinical information, and overall survival. The results indicated that low PTPN2 with high FSP-1 expression correlate with poor survival outcomes in cases of CRC. Next, the *in vitro* and *in vivo* study further indicated that TAFs contribute to the function of PTPN2 in migration of CRC. Mechanically, PTPN2 inhibits metastasis of CRC with TAFs in the JAK/STAT signaling pathway.

Material and methods

Patient tissue samples and clinical information

The study collected of CRC tissue samples from the department of Pathology, Nanjing First Hospital, Nanjing Medical University from January 2009 to December 2014. Total formalin-fixed, paraffin-embedded (FFPE) samples, included 36 colonitis tissues, 562 cancer tissues (CA), 118 paracancerous (PA), and 74 metastatic lymph node tissues (mCA). The corresponding clinical information of tissues from 562 patients, 384 males and 178 females with a median age of 67.41 y (range, 30 to 98 y), tumor location (right, left, transverse, and sigmoid), histological type (adenocarcinoma, mutinous/SRCC), differentiation (well, moderate, poor, and others), T stage (T1-2, T3, and T4), N stage (N0, N1, and N2), M stage (M0 and M1), AJCC stage (I and III-IV), venous invasion (negative and positive), perineural invasion (negative and positive), preoperative CEA (≤ 5 and > 5) Ki67 (negative and positive), and overall survival (OS). No CRC patients received any other therapy before surgery. The study protocol was approved by the Human Research Ethic Committee of Nanjing Medical University.

Clinical TMA construction and immunohistochemistry double-staining

Construction of TMA has been described previously. A total of 11 colon TMAs was constructed. IHC staining was applied to the two markers anti-PTPN2 antibody (BS6716, Bioworld) and anti-S100A4 antibody (CL0239, ThermoFisher). Paraffin sections were prepared for baking at 60°C for 1.5 h. The first antibody PTPN2 uses AP Red for color development, and the second antibody S100A4 uses DAB for color development, Hematoxylin and Bluing reverse blue liquid, Hematoxylin and Bluing liquid, Hematoxylin II and Bluing to back blue, section gradient ethanol dehydration to xylene, and neutral gum seal solid were used.

Cell culture and reagents

The human CRC cell lines HCT116 and LoVo were purchased from KeyGEN BioTECH (Nanjing, China) and cultured in cell medium. Plasmid was transfected using Lipofectamine 2000 (Invitrogen). knock-down lentivirus plasmid that was constructed targeting PTPN2 gene was synthesized by Guangzhou GeneCopoeia Company. The target sequences of knock-downed PTPN2 are listed as follows: sh-PTPN2-1: 5'-GTACAGTGCGACAGCTAGAAT-3'; sh-PTPN2-2: 5'-CGATAATGATGGAAATTAAGT-3'; sh-PTPN2-3: 5'-GCATAGTTGTTACAAGCTACA-3'.

Proliferation and migration assay

To examine the function of PTPN2 on proliferation of CRC cell lines, 3 to 5 × 10^3 cells per well

were seeded in 96-well plates, transfected with different plasmids for 24 h followed by use of BrdUrd incorporation assay (Calbiochem, USA) to detect the proliferation of cells. BrdUrd was added into medium and kept for 10 h, and we measured the absorbance in each well at wavelengths of 450 and 540 nm.

To examine the function of PTPN2 on migration of CRC cell lines, we seeded 3×10^4 cells into the upper well of a Boyden chamber of a 24-well plate and cultured specimens for 48 h. Following transfection with different plasmids, after 48 h, the cells were stained with Hematoxylin and photographed.

Real-time PCR and Western blot assay

Cell RNA was prepared by TRIzol reagent (Invitrogen), and reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit protocol (Takara Bio). qRT-PCR was performed on an ABI 7500HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All reactions were repeat in triplicate. Primers used in the study were as follows: human GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3'; human PTPN2 forward, 5'-GCAGTGAGAGCATTCTACGGA-3' and reverse, 5'-TGACACAAACCCCATCTTAGTGA-3'. Cellular proteins were lysed as previously described. Equal amounts of cell proteins were separated in 8%-10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with TBS containing 0.1% Triton X-100 and 5% non-fat milk for 2 h at RT, then incubated with PTPN2 (1:1000, CST), *p*-JAK1 (1:1000, CST), *p*-JAK2 (1:1000, CST), *p*-JAK3 (1:1000, CST), *p*-STAT4 (1:1000, CST), *p*-STAT6 (1:1000, CST), vimentin (1:1000, Affinity), and GAPDH (1:1000, Santa Cruz) at 4°C overnight, and then incubated with HRP-conjugated anti-IgG at RT for 1 h.

Mouse model

NOD mice were obtained from Beijing Vital River Laboratory Animal Technology. HCT116, HCT116+TAFs, HCT116 shRNA-PTPN2, and HCT116 shRNA-PTPN2+TAFs cell lines were cultured in media and injected (1×10^6 cells in 100 μl PBS) into 4 to 5-week-old mice ($n = 5$ per group) at a site on the chest. The tumor diameter was measured with a Vernier caliper and data recorded every two days. Tumor volume was calculated as follows: $V = 1/2AB^2$, where A represents the longest diameter, and B represents the vertical diameter. Mice were euthanized and the tumors were harvested for immunohistochemistry and hematoxylin and eosin staining.

Statistical analysis

All data statistical analysis was performed using SPSS 23.0 software. *x*-tile software was used to evaluate cut-off points according to CRC patients' clinical pathological information. The study classified the PTPN2 and FSP-1 points into "low or no" and "high". The Pearson x^2 test was used to compare expression level of PTPN2 and FSP-1 in several types of colon tissue samples and the association of target gene expression level with clinical pathological parameters. CRC patients' OS was evaluated with Kaplan-Meier and the log-rank method. Univariable and multivariable Cox proportional hazards regression models were employed to investigate independent prognostic factors. Independent Student's *t*-test was used to compare the mean value of two groups: $P < 0.05$ was considered significant differences.

Results

Low PTPN2 with high FSP-1 expression correlated with poor survival outcomes of CRC

Double-staining IHC assay detected PTPN2 and FSP-1 expression in total 790 CRC samples, including 36 colonitis samples, 118 paracancerous tissue samples (PA), 562 cancer samples (CA), and 74 examples of metastasis of CRC samples (mCA). The expression of PTPN2 was predominantly localized in the cytoplasm of CRC cells (**Figure 1A-D**). The study identified the cut-off scores according to OS of CRC patients. For PTPN2, the cut-off scores of 0-140 as low or no expression, while 141-300 was considered high expression; For FSP-1, the cut-off scores of 0-130 as low or no expression, while 131-300 was considered high expression. High PTPN2 and high FSP-1 expression (P+F+), high PTPN2 and low FSP-1 (P+F-), low PTPN2 and high FSP-1 (P-F+), low expressions of PTPN2 and FSP-1 (P-F-) were detected in colonitis, PA tissues, and in CA and mCA of CRC samples. P-F+ expression was detected in CRC tissues 134/562 (23.80%) compared with that in PA samples 11/118 (9.30%). Interestingly,

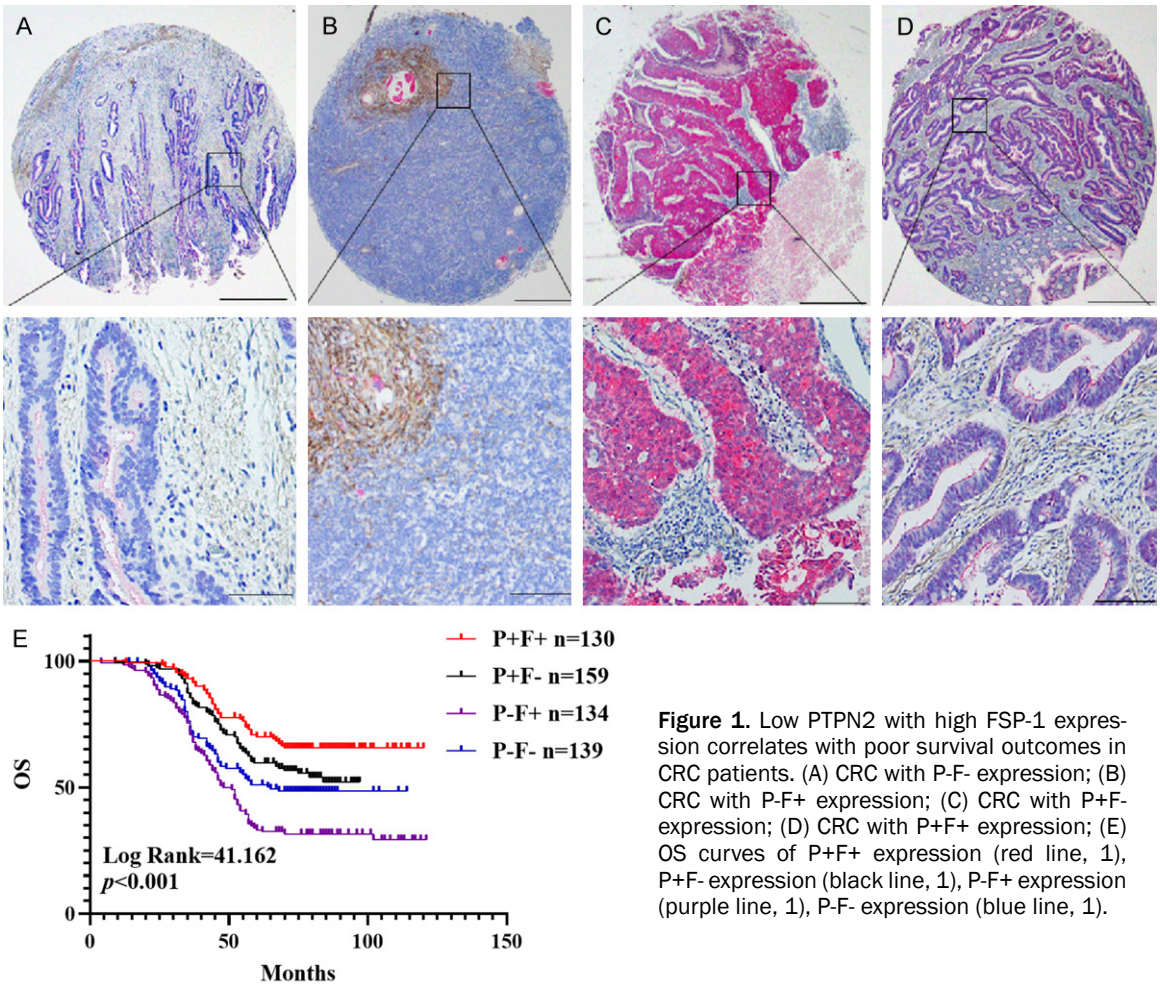


Figure 1. Low PTPN2 with high FSP-1 expression correlates with poor survival outcomes in CRC patients. (A) CRC with P-F- expression; (B) CRC with P-F+ expression; (C) CRC with P+F- expression; (D) CRC with P+F+ expression; (E) OS curves of P+F+ expression (red line, 1), P+F- expression (black line, 1), P-F+ expression (purple line, 1), P-F- expression (blue line, 1).

Table 1. PTPN2 and FSP-1 co-expression in CRC tissues

Characteristics	n	PTPN2	+	+	-	-	*P*	χ^2
		FSP-1	+	-	+	-		
Colonitis	36		12 (33.30)	4 (11.10)	9 (30.60)	11 (30.50)	< 0.001*	69.519
PA	118		17 (14.40)	60 (50.80)	11 (9.30)	30 (25.40)		
CA	562		130 (23.10)	159 (28.30)	134 (23.80)	139 (24.70)		
mCA	74		12 (16.20)	6 (8.10)	35 (47.30)	21 (28.40)		

*$P < 0.05$.

P-F+ expression was detected more frequently in metastasis of CRC 35/74 (47.30%) (**Table 1**).

To find the association of PTPN2 and FSP-1 expression with clinicopathologic characteristics in CRC, IHC assay revealed that P-F+ expression was significantly related to tumor location ($\chi^2 = 20.562$, $P = 0.015$), N stage ($\chi^2 = 24.696$, $P = 0.003$), M stage ($\chi^2 = 24.382$, $P < 0.001$) and Ki-67 ($\chi^2 = 14.411$, $P = 0.002$); however, no significant relationship was found in gender, age, histological type, differentiation, T stage, AJCC stage, venous invasion, perineural invasion, or preoperative CEA (**Table 2**).

To ascertain the relationship between PTPN2/FSP-1 expression and OS of CRC patients, Kaplan-Meier survival curve analyses suggested that the group with P-F+ expression was associated with shorter OS of CRC patients ($\chi^2 = 41.162$, $P < 0.001$) (**Figure 1E**). Furthermore, the results of univariate and multivariate analyses implied that P-F+ protein expression, N

Table 2. The relationship between PTPN2/FSP-1 co-expression and clinicopathologic characteristics in CRC patients

Characteristics	n	PTPN2	+	+	-	-	P	χ^2
		FSP-1	+	-	+	-		
Total	562							
Gender							0.179	4.901
Male	384		89 (23.20)	101 (26.30)	101 (26.30)	83 (24.20)		
Female	178		41 (23.00)	58 (32.60)	33 (18.50)	46 (25.80)		
Age							0.37	3.143
< 60	300		63 (21.00)	93 (31.00)	69 (23.00)	75 (25.00)		
≥ 60	262		67 (25.60)	66 (25.20)	65 (24.80)	64 (24.40)		
Location							0.015*	20.562
Right	208		61 (29.30)	53 (25.50)	44 (21.20)	50 (24.00)		
Left	109		17 (15.60)	27 (24.80)	36 (33.00)	29 (26.60)		
Transverse	189		43 (22.80)	54 (28.60)	46 (24.30)	46 (24.30)		
Sigmoid	56		9 (16.10)	25 (44.60)	8 (14.30)	14 (25.00)		
Histological type							0.705	1.401
Adenocarcinoma	504		116 (23.00)	144 (28.60)	117 (23.20)	127 (25.20)		
Mutinous/SRCC	58		14 (24.10)	15 (25.90)	17 (29.30)	12 (20.70)		
Differentiation							0.266	14.566
Well	81		14 (17.30)	20 (24.70)	21 (25.90)	26 (32.10)		
Moderate	350		87 (24.90)	94 (26.90)	88 (25.10)	81 (23.10)		
Poor	102		26 (25.50)	36 (35.30)	18 (17.60)	22 (21.60)		
Others	28		3 (10.70)	9 (32.10)	7 (25.00)	9 (32.10)		
T stage							0.741	3.526
T1-2	87		18 (20.70)	27 (31.00)	19 (21.80)	23 (26.40)		
T3	385		96 (24.90)	107 (27.80)	89 (23.10)	93 (24.20)		
T4	90		16 (17.80)	25 (27.80)	26 (28.90)	23 (25.60)		
N stage							0.003*	24.696
N0	177		42 (23.70)	47 (26.60)	47 (26.60)	41 (23.20)		
N1	291		70 (24.10)	76 (26.10)	57 (19.60)	88 (30.20)		
N2	94		18 (19.10)	36 (38.30)	30 (31.90)	10 (10.60)		
M stage							< 0.001*	24.382
M0	388		109 (28.10)	111 (28.60)	75 (19.30)	93 (24.00)		
M1	174		21 (12.10)	48 (27.60)	59 (33.90)	46 (26.40)		
AJCC stage							0.745	0.745
I-II	182		43 (23.60)	48 (26.40)	43 (23.60)	48 (26.40)		
III-IV	380		87 (22.90)	111 (29.20)	86 (22.60)	96 (25.30)		
Venous invasion							0.959	0.306
Negative	430		100 (23.20)	123 (28.60)	103 (24.00)	104 (24.20)		
Positive	132		30 (22.70)	36 (27.30.84)	31 (23.50)	35 (26.50)		
Perineural invasion							0.331	3.426
Negative	432		101 (23.40)	115 (26.60)	103 (23.80)	113 (26.20)		
Positive	130		29 (22.30)	44 (33.80)	31 (23.80)	26 (20.00)		
Preoperative CEA, ng/ml							0.83	0.880
≤ 5	302		71 (23.50)	83 (27.50)	76 (29.20)	72 (23.80)		
> 5	260		59 (22.70)	76 (29.20.74)	58 (22.30)	67 (25.80)		
Ki67							0.002*	14.411
Negative	130		25 (19.20)	52 (40.00)	20 (15.40)	33 (25.40)		
Positive	432		105 (24.30)	107 (24.80)	114 (26.40)	106 (24.5)		

*$P < 0.05$.

Table 3. Univariate and multivariate analyses of prognostic factors for 5-year survival in CRC

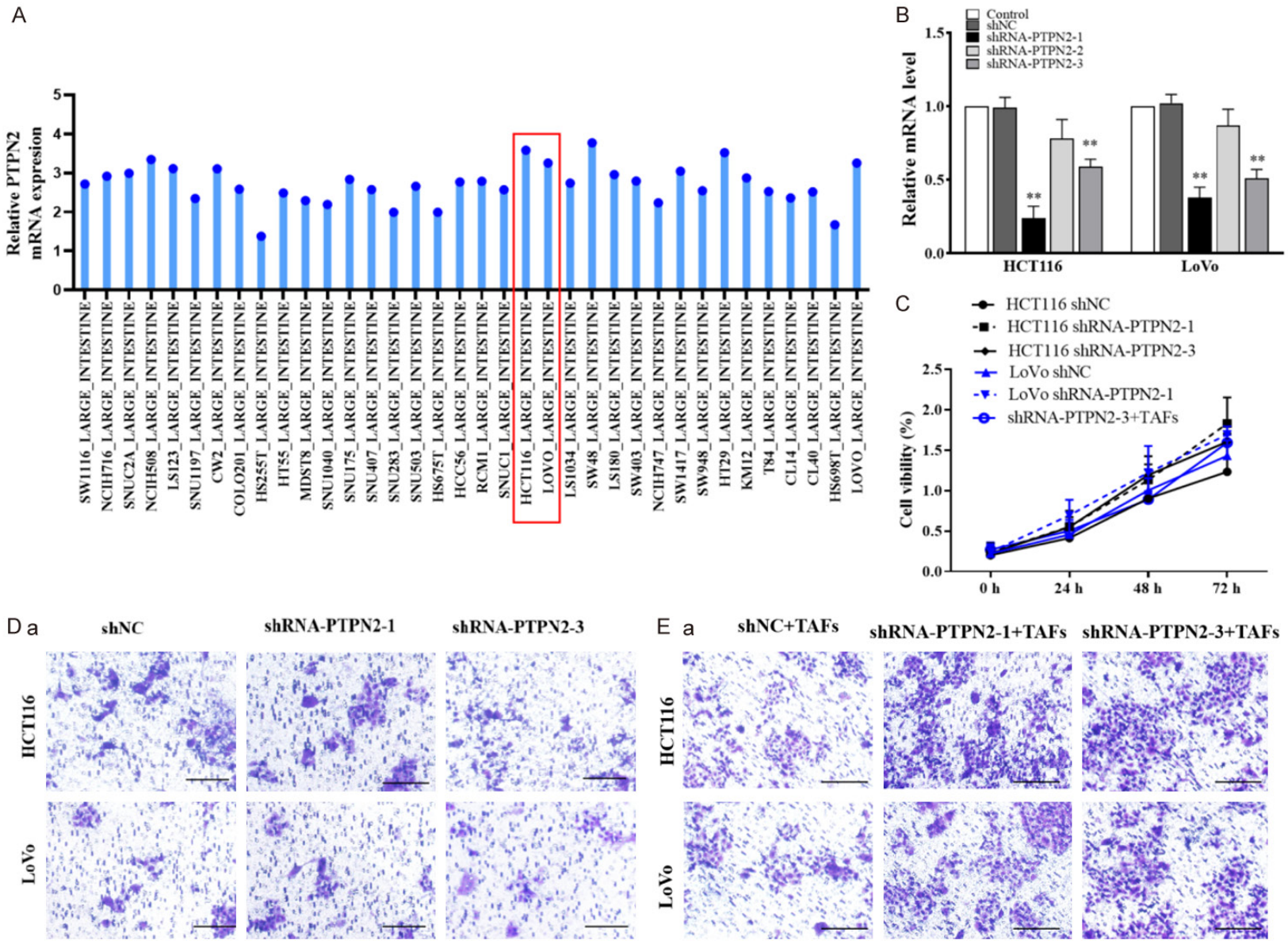
	Univariate analysis			Multivariate analysis		
	HR	*p*-value	95% CI	HR	*p*-value	95% CI
PTPN2 and FSP-1						
P+F+ versus P+F- versus P-F+ versus P-F-	1.308	0.001*	1.122-1.525	1.288	0.004*	1.085-1.530
Age						
< 60 versus ≥ 60	1.113	0.528	0.799-1.55	-	-	-
Gender						
Male versus Female	0.953	0.79	0.668-1.360	-	-	-
Location						
Right versus Transverse versus Left versus Sigmoid	1.077	0.364	0.918-1.263	-	-	-
Histological type						
Adenocarcinoma versus Mutinous/SRCC	0.869	0.613	0.504-1.497	-	-	-
Differentiation						
Well versus Moderate versus Poor versus Others	0.998	0.987	0.794-1.255	-	-	-
AJCC stage						
I-II versus II-IV	3.069	< 0.001*	2.124-4.435	1.663	0.088	0.928-2.980
T stage						
T1-2 versus T3 versus T4	1.495	0.009*	1.107-2.02	1.352	0.098	0.946-1.932
N stage						
N0 versus N1 versus N2 versus N3	3.223	< 0.001*	2.424-4.285	1.677	0.027*	1.061-2.650
M stage						
M0 versus M1	6.557	< 0.001*	4.328-9.935	4.043	< 0.001*	2.441-6.696
Venous invasion						
Negative versus Positive	1.018	0.924	0.627-1.778	-	-	-
Perineural invasion						
Negative versus Positive	1.035	0.865	0.699-1.532	-	-	-
Preoperative CEA, ng/ml						
≤ 5 versus > 5 versus Unknown	1.112	0.531	0.798-1.549	-	-	-
Ki67						
Negative versus Positive	0.406	1.181	0.797-1.751	-	-	-

*$P < 0.05$.

stage, and M stage were significantly associated with poor prognosis (**Table 3**).

Tumor associated fibroblasts (TAFs) contributed the migration function of PTPN2 in CRC

The results of IHC showed that lower expression of PTPN2 and higher expression of TAFs were associated with advanced tumor stage of CRC, therefore, we might design research to detect the function of PTPN2 *in vitro*. We evaluated the level of expression of PTPN2 in CRC cell lines from CCLE (Broad Institute Cancer Cell Line Encyclopedia) database. According to the results of the analysis, the study selected two CRC cell lines (HCT116 and LoVo) with relatively high expression for cytological and animal experiments (**Figure 2A**). Next, the study knocked down PTPN2 expression (shRNA-PTPN2) in HCT116 and LoVo cell lines. The efficiency of shRNA-PTPN2 was detected in changes in mRNA level, and we selected the No. 1 and 3 segments with the best knockdown effect to continue the subsequent functional experiments (**Figure 2B**). Furthermore, we explored proliferation in two cell lines and the results indicated that shRNA-PTPN2 did not inhibit cell growth function (**Figure 2C**). We also found cell migration did not reduced significantly after knocking down PTPN2 in two CRC cell lines (**Figure 2D**). We speculated that the complex tumor microenvironment (TME) in CRC also contributes to the function of PTPN2. TAFs can have extensive cross-talk with cancer cells by secreting cytokines, inflammatory factors, and angiogenic factors, thus playing an important role in tumor cell proliferation, metastasis, and angiogenesis. Therefore, we investigated whether TAFs contribute to the function of PTPN2 in migration of CRC cells. Experiments


A
Relative PTPN2 mRNA expresion
SW1116_LARGE_INTESTINE
NCIH716_LARGE_INTESTINE
SNUC2A_LARGE_INTESTINE
NCIH508_LARGE_INTESTINE
LS123_LARGE_INTESTINE
SNU1197_LARGE_INTESTINE
CW2_LARGE_INTESTINE
COLO201_LARGE_INTESTINE
HS255T_LARGE_INTESTINE
HT55_LARGE_INTESTINE
MDST8_LARGE_INTESTINE
SNU1040_LARGE_INTESTINE
SNU175_LARGE_INTESTINE
SNU407_LARGE_INTESTINE
SNU283_LARGE_INTESTINE
SNU503_LARGE_INTESTINE
HS675T_LARGE_INTESTINE
HCC56_LARGE_INTESTINE
RCM1_LARGE_INTESTINE
SNUC1_LARGE_INTESTINE
HCT116_LARGE_INTESTINE
LOVO_LARGE_INTESTINE
LS1034_LARGE_INTESTINE
SW48_LARGE_INTESTINE
LS180_LARGE_INTESTINE
SW403_LARGE_INTESTINE
NCIH747_LARGE_INTESTINE
SW1417_LARGE_INTESTINE
SW948_LARGE_INTESTINE
HT29_LARGE_INTESTINE
KM12_LARGE_INTESTINE
T84_LARGE_INTESTINE
CL14_LARGE_INTESTINE
CL40_LARGE_INTESTINE
HS698T_LARGE_INTESTINE
LOVO_LARGE_INTESTINE
B
Control
shNC
shRNA-PTPN2-1
shRNA-PTPN2-2
shRNA-PTPN2-3
Relative mRNA level
HCT116
LoVo
C
HCT116 shNC
HCT116 shRNA-PTPN2-1
HCT116 shRNA-PTPN2-3
LoVo shNC
LoVo shRNA-PTPN2-1
shRNA-PTPN2-3+TAFs
Cell vibility (%)
0 h
24 h
48 h
72 h
D a
shNC
shRNA-PTPN2-1
shRNA-PTPN2-3
HCT116
LoVo
E a
shNC+TAFs
shRNA-PTPN2-1+TAFs
shRNA-PTPN2-3+TAFs
HCT116
LoVo

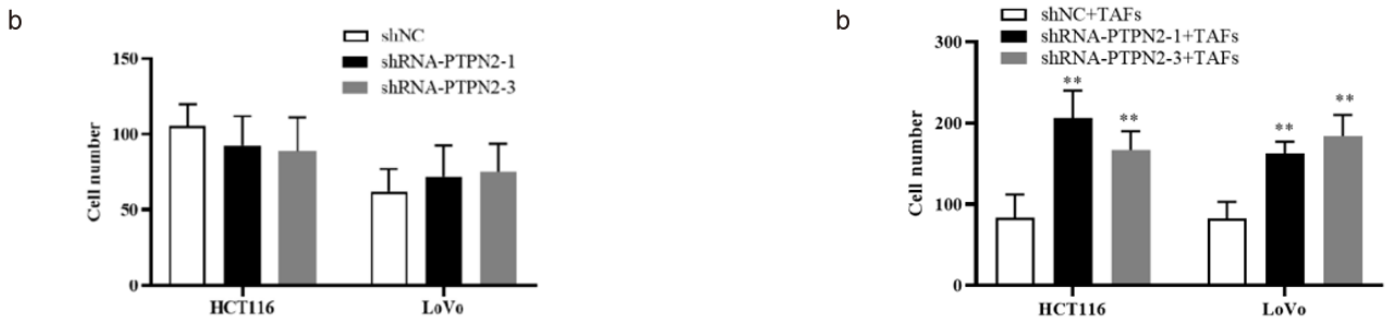


Figure 2. The function of PTPN2 in CRC cell lines. (A) The level of expression of PTPN2 in CRC cell lines from the CCLE database; (B) The efficiency of shRNA-PTPN2 was detected in mRNA level by qRT-PCR assay; (C) The proliferation ability of CRC cell lines after shRNA-PTPN2 were detected by proliferation assay; (D) Effect of PTPN2 knock-down on migration in CRC cell lines, (b) plots statistics pertaining to (a). Mean ± SD, $^{**}P < 0.001$, were compared with control group. (E) Effect of PTPN2 knock-down on migration in CRC cell lines with TAFs, (b) plots statistics pertaining to (a). Immunostaining (400×); scale bar, 50 mm.

involving a co-culture system through trans-well migration assay. We added hCAFs (HELF) to the lower chamber, and added HCT116 shNC, HCT116 shRNA-PTPN2, LoVo shNC, and LoVo shRNA-PTPN2 in the upper chamber, respectively. The results found that TAFs induced a number of shRNA-PTPN2 CRC cells to migrate, compared with the group containing shNC (**Figure 2E**).

PTPN2 inhibited metastasis of CRC with TAFs in the JAK/STAT signaling pathway

To investigate whether TAFs participate the function of PTPN2 in CRC cells, we detected the level of TGF-β1 (**Figure 3A**) in the supernatants by ELISA. We found co-cultured CRC cells and TAFs could increase the level of TGF-β1, when compared with CRC cells group. And the level of TGF-β1 in TAFs and shRNA-PTPN2 group were higher than that in the TAFs and shNC groups.

To assess the mechanism of PTPN2 regulate migration with TAFs, we conducted STRING database (https://string-db.org/cgi/network.pl?taskId=AZxIoAbSz7Ad) searches to explore the interactions between PTPN2 and FSP-1 (**Figure 3B**). The results revealed that lower PTPN2 expression might cross-talk with FSP-1 through the JAK-STAT signaling pathway, thus inducing EMT. To characterize the correlation between PTPN2 and FSP-1, we detected the mRNA (**Figure 3C**) and protein (**Figure 3D**) expression level of the JAK-STAT signaling pathway and EMT marker after shRNA-PTPN2 action in CRC cells in co-cultured cell lines. Western blot results indicated that phosphop-JAKs-STATs increased significantly after the action of shRNA-PTPN2 in CRC cells, moreover, vimentin expression was also increased. The mRNA level is same as that of the protein expression. These results indicated that the JAK/STAT signaling pathway was critical for PTPN2-TAFs-mediated migration of CRC cells.

TAFs contributed the function of PTPN2 in colorectal carcinogenesis in vivo

To assess the mechanism of PTPN2 *in vivo*, we used immunodeficient mice harboring tumors derived from HCT116, HCT116+TAFs, HCT116 shRNA-PTPN2, and HCT116 shRNA-PTPN2+TAFs cell lines. After four weeks of, mice bearing HCT116 shRNA-PTPN2+TAFs tumors showed an increased level of tumor growth compared with other groups (**Figure 4A**, **4B**). To analyze the regulatory mechanism *in vivo*, we detected the protein level of PTPN2, Ki67 and vimentin. In the HCT116 shRNA-PTPN2+TAFs group, there were more infiltrating cells between tumor cells (**Figure 4C**).

Discussion

Tyrosine phosphorylation is an important mechanism underpinning the regulation of signal transduction, which plays a key role in a series of physiological processes [11, 12]. In the present study, we aimed to evaluate the relationship of a protein tyrosine phosphatase PTPN2 with metastasis of CRC. PTPN2 was down-expressed in CRC tissue samples, compared with other types of colon tissues. In the CRC TMAs, we also collected 74 cases of lymph node metastasis tissues. Through double staining assay, the study provides a unique perspective from which to observe the regulatory role of PTPN2 in the CRC tumor microenvironment. From double-staining data, we found that the expression of PTPN2-FSP-1+ was more frequent in metastasis of CRC 35/74 (47.30%), even higher than in CRC tissues (134/562 (23.80%)), PA samples (11/118 (9.30%)), and colonitis tissues (9/36 (30.60%)). These findings concurred with previous data revealing that tyrosine phosphorylation is also involved in coordinating adjacent cell interactions during these processes, such as mediating soluble factors with cells or direct cell-cell interactions in the immune system [13]. We also found that P-F+ expression was significantly related to tumor location, N stage, M stage, and Ki-67 by IHC assay. The results of Kaplan-Meier survival curves indicated that the group with T-F+ expression was associated with shorter OS of CRC patients. The results of univariate and multivariate analyses further confirmed this view.

We found that the knockdown of PTPN2 did not influence the proliferation and migration ability of CRC cell lines, therefore, we constructed cell models about the co-cultured CRC cell lines and TAFs and found TAFs contribute to the migration function of PTPN2 in CRC. A new study published in *Nature Immunology* by W. Nicholas Haining and Arlene H. Sharp of Harvard Medical School in 2019, showed that PTPN2 can regulate the production of depleted CD8+ positive T cell subsets and control tumor immunity. Meanwhile, several studies also confirmed the expression of PTPN2 in cancer cells

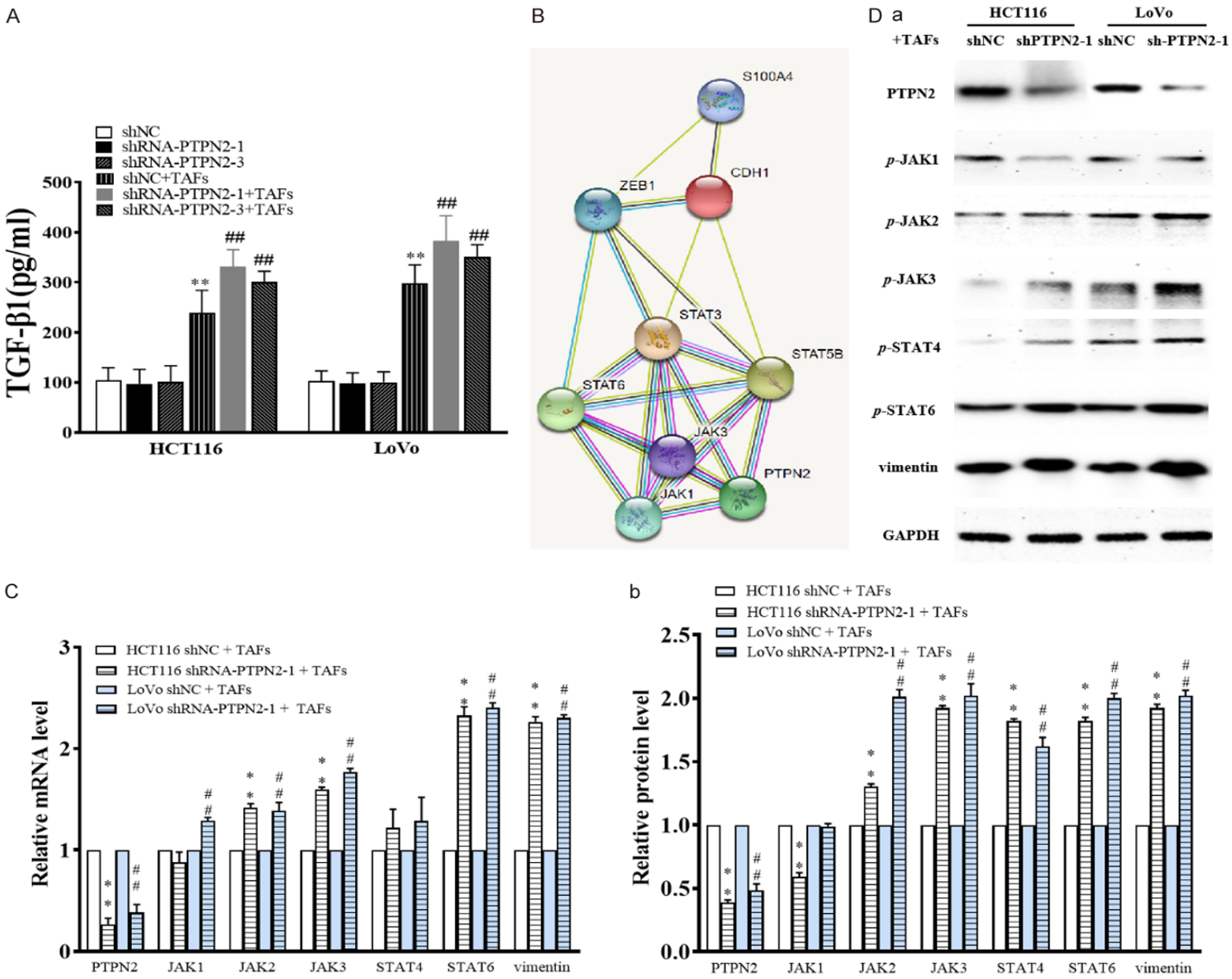

A
shNC
shRNA-PTPN2-1
shRNA-PTPN2-3
shNC+TAFs
shRNA-PTPN2-1+TAFs
shRNA-PTPN2-3+TAFs
TGF-β1(pg/ml)
HCT116
LoVo
B
S100A4
CDH1
ZEB1
STAT3
STAT5B
STAT6
JAK3
PTPN2
JAK1
D a
HCT116
LoVo
+TAFs
shNC
shPTPN2-1
shNC
sh-PTPN2-1
PTPN2
p-JAK1
p-JAK2
p-JAK3
p-STAT4
p-STAT6
vimentin
GAPDH
C
HCT116 shNC + TAFs
HCT116 shRNA-PTPN2-1 + TAFs
LoVo shNC + TAFs
LoVo shRNA-PTPN2-1 + TAFs
Relative mRNA level
PTPN2
JAK1
JAK2
JAK3
STAT4
STAT6
vimentin
b
HCT116 shNC + TAFs
HCT116 shRNA-PTPN2-1 + TAFs
LoVo shNC + TAFs
LoVo shRNA-PTPN2-1 + TAFs
Relative protein level
PTPN2
JAK1
JAK2
JAK3
STAT4
STAT6
vimentin

Figure 3. PTPN2 inhibits metastasis of CRC with TAFs in the JAK/STAT signaling pathway. The levels of TGF-β1 (A) in the supernatants were detected by ELISA. (B) STRING database used to analyze the interactions between PTPN2 and FSP-1. qRT-PCR (C) and Western blot (D) to detect the JAK-STAT signaling pathway, Mean ± SD, $^{**}P < 0.001$, were compared with HCT116 shNC+TAFs control group; $^{\#\#}P < 0.001$, were compared with LoVo shNC+TAFs control group.

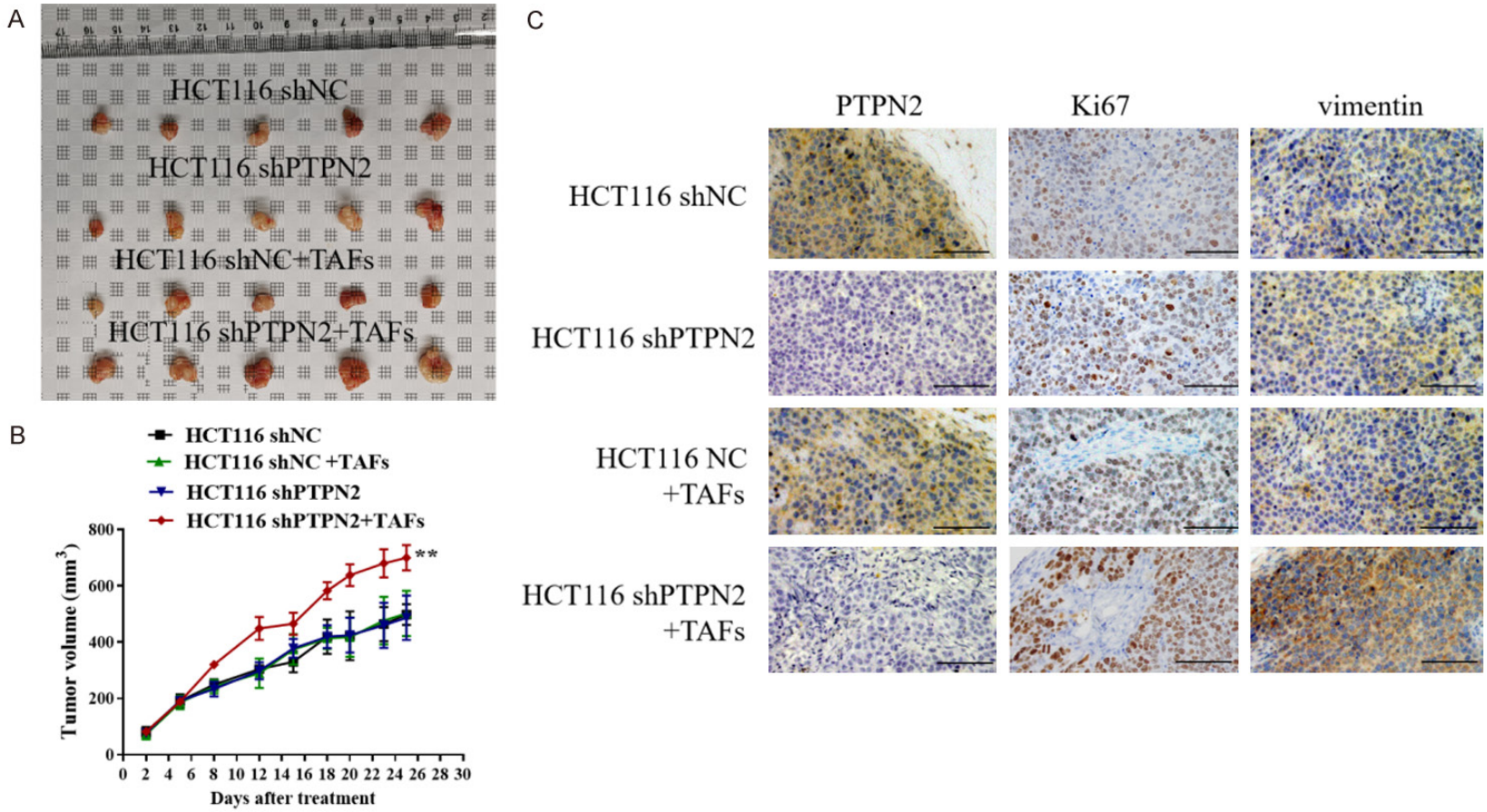


Figure 4. PTPN2 inhibits metastasis of CRC *in vivo*. (A, B) Representative tumors were harvested, each animal was monitored for changes in body mass and tumor mass, $^{*}P < 0.05$, $^{**}P < 0.001$ by two-sided Student's t-test; (C) Paraffin sections of some xenograft tumors were immunostained with several antibodies to verify the protein expression of PTPN2, Ki67, and vimentin. Immunostaining (400×); scale bar, 500 μm.

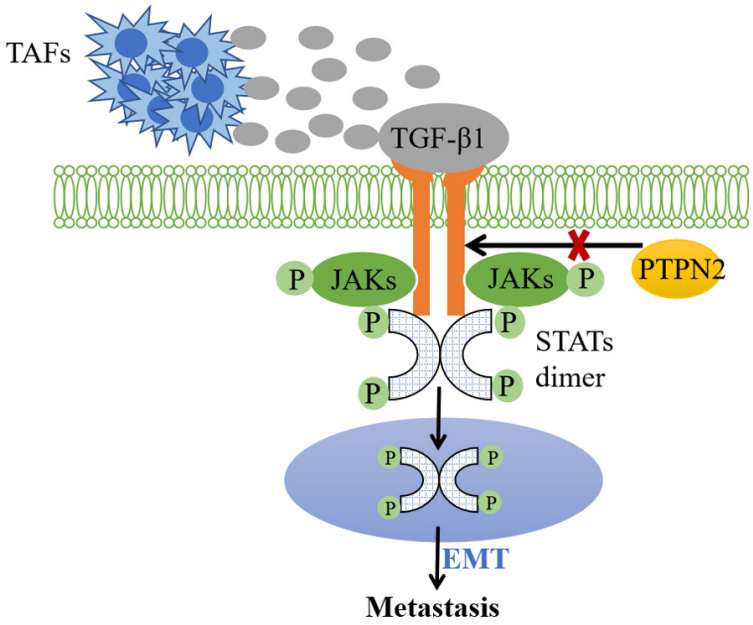


Figure 5. The mechanism of PTPN2 inhibits metastasis of CRC with TAFs in the JAK/STAT signaling pathway.

[5]. We hypothesized that the effect of PTPN2 on tumors must be related to cross-talk between tumor cells and immune cells. In our study, we first focus on the relationship function of PTPN2 in TMEs: the results *in vitro* confirmed our hypothesis. We further detected the supernatants of co-cultured, the results indicated that PTPN2 and TAFs contribute to the migration ability of CRC cells through increases in the levels of TGF-β1.

PTPN2 substrates are various, including IR, the CSF-1 receptor, EGFR, SFKs, JAK1, JAK3, STAT1, STAT5, STAT6, *etc*. [14-17]. Inactivation of PTPN2 is found in T cell acute lymphoblastic leukemia (T-ALL) [18]. The inactivation of PTPN2 causes an increase in JAK-STAT signaling, which in turn affects the malignancy of the tumor [19, 20]. In some breast cancers, the expression of PTPN2 is decreased, while PTPN2 can inhibit the growth of breast cancer cells after re-transplantation of PTPN2, which may be caused by inhibition of the acidification of SFK kinase and STAT3 [6, 13].

The results of STRING database analysis reported that lower PTPN2 expression might cross-talk with FSP-1 to induces EMT phenomenon though the JAK-STAT signaling pathway. We used WB and qRT-PCR assay to verify the results of database information analysis. And found JAK/STAT signaling pathway was critical for PTPN2-FSP-1-mediated migration of CRC cells. Lastly, we also found TAFs contributed the function of PTPN2 in CRC *in vivo*.

Taken together, the study indicated that low PTPN2 with high FSP-1 expression correlate with poor survival of CRC; TAFs contributed the function of PTPN2 in colorectal carcinogenesis through activating JAK/STAT signaling pathway (**Figure 5**). More importantly, double-staining assay could provide us with a unique perspective from which to observe the tumor microenvironment.

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Disclosure of conflict of interest

None.

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