ORIGINAL ARTICLE

Title:

Significance of polypyrimidine tract binding protein 1 expression in colorectal cancer

Authors:

Hidekazu Takahashi¹, Junichi Nishimura¹, Yoshinori Kagawa¹,², Yoshihiro Kano³,
Yusuke Takahashi¹,⁴, Xin Wu¹, Masayuki Hiraki¹, Atsushi Hamabe¹, Masamitsu Konno³,
Naotsugu Haraguchi¹, Ichiro Takemasa¹, Tsunekazu Mizushima¹, Masaru Ishii², Koshi
Mimori⁴, Hideshi Ishii³, Yuichiro Doki¹, Masaki Mori¹, Hirofumi Yamamoto¹

Authors’ affiliations:

¹Department of Gastroenterological Surgery, Osaka University Graduate School of
Medicine, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan

²Laboratory of Cellular Dynamics, WPI-Immunology Frontier Research Center, Osaka
University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan

³Department of Frontier-Science for Cancer and Chemotherapy, Graduate School of
Medicine, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan

⁴Department of Molecular and Cellular Biology, Division of Molecular and Surgical
Oncology, Kyushu University, Medical Institute of Bioregulation, Tsurumihara 4546,
Beppu, Ohita 874-0838, Japan
Running Title

Significance of PTBP1 in CRC

Key words: polypyrimidine tract binding protein 1, cell invasion, cell proliferation, cell cycle regulation, colorectal cancer

Abbreviations list

PTBP1, polypyrimidine tract-binding protein; CRC, colorectal cancer; PKM, pyruvate kinase; CAIX, carbonic anhydrase 9; IRES, internal ribosomal entry site

Footnotes

1) Financial Support

None

2) Corresponding author:

Hirofumi Yamamoto

Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Yamadaoka 2-2, Suita, Osaka 565-0871, Japan

Phone: +81 6 6879 3251; Fax: +81 6 6879 3259

E-mail address: hyamamoto@gesurg.med.osaka-u.ac.jp

3) Potential conflicts of interest

The authors have declared that no conflict of interest exists.

4) Other notes
ABSTRACT

Polypyrimidine tract-binding protein (PTBP1) is an RNA-binding protein with various molecular functions related to RNA metabolism and a major repressive regulator of alternative splicing, causing exon skipping in numerous alternatively spliced pre-mRNAs. Here, we have investigated some role of PTBP1 in colorectal cancer (CRC). PTBP1 expression levels were significantly overexpressed in cancerous tissues than corresponding normal mucosal tissues. We also observed that PTBP1 expression levels, c-MYC expression levels and PKM2/PKM1 ratio were positively correlated in CRC specimens. Moreover, PTBP1 expression levels were positively correlated to poor prognosis and lymph node metastasis. In analyses in CRC cells using siRNA for PTBP1, we observed PTBP1 affects in cell invasion which was partially correlated to CD44 splicing and this correlation was also confirmed in clinical samples. PTBP1 expression
also affected anchorage-independent growth in CRC cell lines. PTBP1 expression also
affected cell proliferation. Using time-lapse imaging analysis, PTBP1 implicated in
prolonged G2/M phase in HCT116 cells. As for the mechanism of prolonged G2/M
phase in HCT116 siPTBP1 cells, western blotting revealed that PTBP1 expression level
was correlated to CDK11\textsuperscript{p58} expression level which was reported to play an important
role on progression to complete mitosis. These findings indicated that PTBP1 is a
potential therapeutic target for CRC.
INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related death in the western world and is estimated to be one of the most frequently diagnosed cancers; estimated new cases of CRC were 142,820 and corresponding expected mortality was 50,830 in the United States in 2013(1). Although monoclonal antibodies, including bevacizumab, an inhibitor of vascular endothelial growth factor, and cetuximab, an epidermal growth factor receptor inhibitor (2, 3), are currently feasible as novel molecular-based therapies, many patients with CRC still die from disease recurrence, mainly because of liver metastasis. Therefore, further elucidation of the molecular mechanisms of CRC is essential for developing novel therapeutic strategies.

Alternative splicing greatly affects protein levels and functions. Cancer-specific abnormal pre-mRNA splicing can affect tumor initiation and promotion (4). Alternative RNA splicing can greatly affect protein levels and functions. In cancer, abnormal splicing often leads to cancer-promoting splice variants that are translated into oncogenes or aberrant tumor suppressors (5). Normal splicing patterns can be disrupted by either cis-acting mutations of splicing regulatory elements (5).

Increases in the splicing factor Polypyrimidine Tract Binding protein (PTBP1, also known as hnRNPI) that are associated with glioma malignancy could have similar...
oncogenic effects (6). PTBP1 has been reported to play a key role in pre-mRNA splicing in cancer. Actually, PTBP1 have a critical effect on pyruvate kinase (PKM) alternative splicing in glioma, and this splicing strongly influences cancer progression (7, 8). PTBP1 also has multiple functions other than pre-mRNA splicing and affects glioma cell invasion (7). In ovarian cancer, PTBP1 levels correlate with the degree of malignancy (9). Higher amounts of PTBP1 occur in advanced, as compared to benign, ovarian tumors and PTBP1 increases when ovarian epithelial cells are immortalized (9). Removal of PTBP1 from ovarian tumor cells makes cell less proliferated, anchorage-independent growth, and cell invasion. Moreover, PTBP1 can form complexes with focal adhesion-encoding transcripts at the cell membrane, which might affect cell spreading (10). When taken together, deregulation of PTBP1 could cause multiple changes in gene expression and translation to promote cancer and targeting PTBP1 might be a potential therapeutic target. However, few findings about PTBP1 have been reported in CRCs.

Here we studied the clinicopathological significance of PTBP1 expression and found that it was associated with lymph node metastasis and invasion as well as with disease-free survival. We evaluated the significance of other related factors, including
tumor invasion and proliferation abilities, which are directly involved in tumor
malignant potentials.

MATERIALS AND METHODS

Patients and sample collection

A total of 178 patients with CRC who underwent surgical treatment at the Kyushu
University at Beppu and affiliated hospitals between 1992 and 2002 were enrolled in
this study. Resected tumors and paired non–tumor tissue specimens were immediately
taken from resected colons and placed in RNAlater (Takara, Japan), embedded in Tissue
Tek OCT medium (Sakura, Tokyo, Japan), or frozen in liquid nitrogen and kept at
−80°C until RNA extraction. The median follow-up period was 2.93 years. All data
pertaining to the samples, including age, sex, tumor size and depth, lymphatic invasion,
lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination,
distant metastasis, clinical stage, and histological grade, were taken from clinical and
pathological records. Written informed consent was obtained from all patients in
accordance with the guidelines approved by the Institutional Research Board. This study
was conducted under the supervision of the ethical board of Kyushu University.

Cell culture
The human colon cancer cell lines HCT116, DLD1, SW480, and HT29 were obtained from the American Type Culture Collection (Manassas, VA) in 2001. Stocks were prepared after passage 2 and stored in liquid nitrogen. All experiments were performed with cells of passage of <8. These cell lines were authenticated by morphologic inspection, short tandem repeat profiling, and Mycoplasma testing by ATCC. Mycoplasma testing was done also by the authors. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO₂.

**RNA preparation for reverse-transcription–PCR**

Total RNA was isolated using a modified acid–guanidinium–phenol–chloroform procedure. Complementary DNA was synthesized from 1 μg of total RNA using random hexamer primers and M-MLV reverse transcriptase (RT) (Invitrogen).

**Evaluation of gene expression in clinical samples**

For quantitative real-time RT (qRT)-PCR, the primer sequences used are listed in Supplemental Table S1. For confirmation of RNA quality, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene served as an internal
control. The amplification protocol included initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s and at 60°C for 30 s. PCR was performed in a LightCycler™ 480 System (Roche Applied Science) using the LightCycler® 480 Probes Master kit (Roche Applied Science). All concentrations were calculated relative to the concentration of cDNA from Human Universal Reference total RNA (Clontech).

**Immunohistochemistry**

Immunohistochemical analyses of PTBP1 were performed using surgical specimens from selected patients with CRC at Osaka and Kyushu University as previously described (11). Briefly, after deparaffinization, antigen retrieval in citrate buffer (pH = 6.0), and blocking according to standard protocols, the antigen–antibody reaction was carried out overnight at 4°C. Mouse monoclonal antibody against human PTBP1 (H00005725-M01, Abnova, Walnut, CA) was used at 3 μg/ml concentration, as was anti-human carbonic anhydrase 9 (CAIX) antibody (3829-1, Epitomics, Burlingame, CA) at a 1:250 dilution. For the enzyme antibody technique, the avidin–biotin–peroxidase method (Vectastain Elite ABC reagent kit; Vector) was used according to standard protocols. Nuclei were stained with hematoxylin. For immunoﬂuorescence evaluation, Alexa Fluor 488–conjugated goat anti-mouse IgG (1:500) and Alexa Fluor
555–conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) (1:1000) were used as secondary antibodies. Nuclei were stained with 4’, 6-diamidino-2-phenylindole–containing mounting medium (Invitrogen).

Assessment of tumor budding

Tumor budding was estimated according to the definition proposed by Ueno et al. (12). An isolated cancer cell or a cluster composed of fewer than five cancer cells was defined as tumor budding. The number of buddings was counted under a high-power field (×200) in the invasive front area.

Transfection of small interfering RNA (siRNA)

The siRNA for PTBP1 (Stealth Select RNAi, HSS143520) and negative control siRNA (Negative Control Hi GC) were purchased from Invitrogen. siRNA for cMYC and negative control (siGENOME non-targeting siRNA) were purchased from Thermo Scientific (Waltham, MA). Cells were transfected with siRNA in 20 nM concentration using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocols.
**Invasion assay**

The invasion assay was performed using Transwell cell culture chambers (BD Biosciences, Bedford, MA) according to the manufacturer’s protocols. Briefly, $5 \times 10^5$ cells were seeded in triplicate on the matrigel-coated membrane. After 48 h, cells that had invaded the undersurface of the membrane were fixed with 100% methanol and stained with 1% toluidine blue. Four microscopic fields were randomly selected for cell counting.

**Proliferation assay**

A total of $5 \times 10^5$ cells were seeded onto 6-well plates (BD). At 24 h after seeding, cells were transfected with siRNAs using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. At 24 h after transfection, $3 \times 10^3$ cells were seeded onto a 96-well plate (BD), and relative cell numbers were determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and the indicated time course.

**Western blotting**

Antibodies were purchased as follows: anti-PTBP1 and anti-Fbwx7 from Abnova; p27, Skp2, cyclin A, cyclin B, cyclin E, CDK2, CDK4, CDC2, and CDC25A from Santa
Cruz Biotechnology; p21 from Cell Signaling Technology; cMyc and cyclin D from Epitomics; CDK11 from Abcam and β-actin from Sigma-Aldrich. Western blotting was carried out as described previously (13). Briefly, the protein samples (15 μg) were separated by 12.5% polyacrylamide gel electrophoresis followed by blotting on a 0.4 μm membrane. After the blocking, the membrane was incubated in the appropriately diluted primary antibody solution. After incubation with secondary antibody solution, protein bands were detected with the Amersham ECL Detection System (Amersham Biosciences, Piscataway, NJ).

Establishment of Fucci-expressing HCT116

mAG-hGem(1/110) and mKO2-hCdt1(30/120) (provided by Dr. Miyawaki, RIKEN-BSI, Japan) (14) were cloned into the CSII-EF-MCS vector (provided by Dr. Miyoshi, RIKEN-BRC, Japan) and transfected into HEK293T cells with packaging plasmids (15). Stable transformants were selected by FACS Aria (BD). mAG and mKO2 were excited by 488-nm laser lines, and their emission detected with 530/30BP or 585/42BP filters, respectively.

Time-lapse imaging of Fucci-expressing HCT116
A total of $5.0 \times 10^4$ Fucci-expressing HCT116 cells treated with transfection of siRNA (control or anti-PTBP1) were spread into each chamber of a 4-well chamber glass-bottom dish (CELLview, #627871, Greiner Bio One) on the day before observation. Time-lapse imaging was taken with an inverted microscope (A1R; Nikon) with a dry objective lens (Plan-Apo VC, 20X/N.A. 0.75, Nikon). Culture conditions were controlled at 5% CO$_2$ and 37°C with an incubation chamber (INUB-TIZB-F1; TOKAI HIT). mAG and mKO2 were excited by 488-nm and 561-nm laser lines, and their emission detected with 525/25BP or 595/25BP filters, respectively. The images were taken every other hour for 48 h at 20 points for each sample. Raw imaging data were processed with Imaris (Bitplane) with a Gaussian filter for noise reduction. Automatic object counting with Imaris Spots was aided by manual correction to retrieve cell coordinates over time.

**Colony formation assay**

$2\times10^5$ cells were transfected with siRNAs in 6 well plates. After 48 hours culture, cells were trypsized and 5000 cells were re-seeded into each well of CytoSelect were re-seeded into each (Cell Biolab INC, San Diego, CA). After additional 7 days culture, cell dose was obtained, according to the manufacturer's protocols, from
absorbance (485/520 nm) using standard multiplate reader (PerkinElmar INC, Kanagawa, Japan).

Antitumor activity assay

Seven-week-old BALB/cA nude mice were purchased from CLEA Japan (Tokyo, Japan). HCT116 (1.0 × 10⁶ cells) were inoculated subcutaneously in both the left and right flanks of the mice to prepare the solid tumor model. An antitumor activity study was performed when the HCT116 tumors were 5–6 mm in diameter. Mice were treated on days 0, 2, 4, 7, 9, and 11 with 20 µg of sApa-control-siRNA or sApa-PTBP1-siRNA. Antitumor activity was evaluated in terms of tumor size, which was estimated using the following equation: \( V = a \times b^2/2 \), where \( a \) and \( b \) represent the major and minor axes of the tumor, respectively.

Statistical analysis

Statistical analyses were performed using JMP 8.0.1 for Windows (SAS Institute, Cary, NC). Possible differences between groups were analyzed using Student’s t-test, \( \chi^2 \) test, Wilcoxon’s test, or repeated-measures ANOVA. Survival curves were obtained by the Kaplan–Meier method; comparison between curves was completed with the log-rank test.
test. A probability level of 0.05 was chosen to indicate statistical significance.
RESULTS

PTBP1 is preferentially overexpressed in clinical CRCs

A total of 178 paired normal mucosa and primary tumor samples were studied using qRT-PCR. The expression value of PTBP1 mRNA in tumor tissues was significantly higher than that for corresponding paired normal tissues ($P < 0.0001$, t-test; Supplemental Figure S1A). Supplemental Figure S1B shows the results of immunohistochemical studies of PTBP1 expression in representative clinical samples of normal mucosa (a), well-differentiated adenocarcinoma (b), moderately differentiated adenocarcinoma (c), and poorly differentiated adenocarcinoma (d). The majority of the PTBP1 expression was observed in cancer cells, the minority in stromal cells, and scarce positivity in normal colonic epithelium. Immunohistochemical studies revealed that the staining was strong ($n = 7$), moderate ($n = 9$), or weak ($n = 18$) in the tumor cells but very weak or nonexistent in the normal cells in all 34 cases. Immunohistochemical staining intensity differed significantly between the tumor and the normal samples ($P < 0.01$, data not shown), and the data were similar to those obtained from mRNA expression analysis. All 16 tumors with strong or moderate immunohistochemical expression for which RNA data also were available showed higher mRNA expression values (Supplemental Figure S2; $P = 0.012$). The expression
of PTBP1 mRNA seemed to correlate with protein expression.

**PTBP1 expression is correlated with c-MYC expression**

PTBP1 is transcriptionally upregulated by the Myc oncoprotein in glioma cells (8). To assess if this transcriptional regulation exists in CRC, we performed qRT-PCR for Myc in 64 clinical CRC specimens and identified a relationship between PTBP1 and MYC mRNA levels (Supplemental Figure S3A; $P = 0.018$). In CRC cell lines, silencing Myc with siRNA reduced PTBP1 protein levels (Supplemental Figure S3B).

**PTBP1 regulates PKM isotype switch in CRC**

As reported in gliomas (8), we hypothesized that PTBP1 regulates PKM isotype in colon cancer. To investigate our hypothesis, we analyzed human normal mucosa ($n = 3$) and clinical cancer specimens ($n = 10$). RT-PCR including exons 8–11 was performed, followed by digestion with PstI. If exon 10 was included (PKM2), the PCR products were divided into two fragments (Figure 1A). Normal mucosa samples had both PKM1 and PKM2 isoforms; by contrast, cancerous tissues were PKM2 dominant except in one case (Figure 1B). Fluorescence co-immunohistochemistry for PTBP1 and PKM2 of clinical specimen revealed that in tumor surface both of them were slightly positive...
(Figure 1C left panel), in invasive front both of them were ubiquitously positive (Figure 1C right panel) and the distribution of both molecule was tightly overlapped suggested a relationship between these molecules in clinical specimens.

Next, we performed silencing of PTBP1 using transient siRNA in four CRC cell lines (HCT116, DLD1, HT-29, and SW480). As indicated in Figure 2A, the inclusion ratio of PKM1 went up with PTBP1 silencing. Immunohistochemistry showed that PTBP1-positive lesions had a tendency to be distributed in the tumor invasive front (Figure 1C). Next, we analyzed early stage cancers by immunohistochemistry, dividing samples into two groups according to the grade of staining (Supplemental Figure S4). In the strongly positive group, lymphatic invasion, venous invasion, and number of buddings were significantly higher than the negative/weakly positive group (Table 1).

Because hypoxia promotes lymph node metastasis (16, 17), we performed co-immunostaining for PTBP1 and CAIX in the early stage clinical specimens. PTBP1-positive cases (Figure 2B left panel) had no CAIX-positive lesions in the surface or middle layer (Figure 2B (a), (b)), however, focal CAIX positive lesions were observed in the invasive front which PTBP1 was highly positive (Figure 2B (c)); in contrast, PTBP1-negative cases had no CAIX-positive lesions even in the invasive front (Figure 2B right panel and Figure 2B (d)).
**PTBP1 expression is associated with tumor invasion through CD44 splicing**

Because CD44 splicing variants in CRC, especially variant v8-10, are reported to have a strong relationship with the tumor invasion (18, 19), we hypothesized that PTBP1 regulates CD44 splicing in CRC. To assess this issue, we performed qRT-PCR of 91 clinical specimens using a CD44v8-10–specific primer pair. The PTBP1 high-expression group also had significantly higher expression of CD44v8-10 mRNA than did the PTBP1 low-expression group (Figure 3A).

Next, we analyzed four cell lines using another primer pair (Supplemental Figure S5A) for validation of CD44 variant expression. By gel electrophoresis (Supplemental Figure S5B) and sequence analysis (Supplemental Figure S5C), CD44v8-10 mRNA was positive in HCT116, DLD1, HT29, and SW480, and CD44v10 mRNA was positive in DLD1 and HT-29, respectively; no other CD44 splicing variants were detected in this analysis. Then, we designed the CD44v8-10–specific primer pair (inside V8-9) with which all reported CD44 splicing variants (total CD44) can be detected (inside exons 2–3). By qRT-PCR, when silencing PTBP1, CD44v8-10 and total CD44 expression ratio were decreased in HCT116 and DLD1 (Figure 3B; Supplemental Figure S5B). In the in vitro invasion assay, silencing PTBP1 led cells to be less invasive in HCT116, DLD1, HT29, and SW480 (Figure 3C). Calculated invaded cells ratio was also significantly
reduced in silencing PTBP1 in each cell lines (Figure 3D).

*PTBP1 promotes cell proliferation* in vivo and in vitro

PTBP1 is reported to promote cell proliferation in embryonic stem cells (20) and glioblastoma cells (7). To assess the effect of PTBP1 in proliferation of CRC cells, we performed a proliferation assay. When silencing PTBP1, proliferation ability was significantly reduced in HCT116, DLD1, HT29, and SW480 (Figure 4A). To further investigate PTBP1 function for proliferation, we performed western blotting for cell cycle mediators. Although it has been reported that PTBP1 modulates the G1-to-S transition through enhancement of IRES-dependent translation of p27kip1 in 293T cells (21), the protein level of p27kip1 in PTBP1-silencing cancer cells was not different from that in control siRNA cells except SW480 cells (Figure 4B).

Interestingly, other various cell cycle mediators including cyclin A, cyclin B, cyclin C, cyclin E, CDC2, and Skp2 were increased with PTBP1 silencing (Figure 4B). To assess this discrepancy, we performed time-lapse imaging on HCT116/Fucci cells. Actually, the G2/M population (green cell) ratio was significantly increased in siPTBP1 (Figure 4C, \( P < 0.0001 \)). To synchronize the cell cycle, we sorted Red (G1/S) cells. In cell cycle analysis, G1/S cells changed into G2/M in a rapid manner with
siPTBP1 (Figure 5A). For further analysis to explain the prolonged G2/M in HCT116 siPTBP1 cells, we performed immunoblotting of whole cell lysate for CDK11\(^{p58}\) which is reported to play an important role for maintenance of sister chromosome cohesion (22). As a result, in HCT116 and DLD1 cells, when silencing PTBP1, CDK11\(^{p58}\) protein levels were down-regulated (Figure 5B). In clinical samples, PTBP1 expression was related to the expression of CDK11\(^{p58}\) protein levels (Supplemental Figure S6). In concert with these findings, PTBP1 might have an important role in progression to complete mitosis of cells though CDK11\(^{p58}\) regulation, in other words, silencing PTBP1 leads to prolonged G2/M phase and cell cycle mediators might be up-regulated by some feedback mechanism, in these settings.

Based on the fact that PTBP1 expression levels have relationship to cell invasion and proliferation, we hypothesized that anchorage independent cell growth depends on PTBP1 expression and we performed colony formation assay. As a result, colony formation activity was down-regulated by silencing PTBP1 in HCT116, DLD1, HT29 and SW480 cells (Figure 5C). We further tested the efficacy of PTBP1 silencing on tumor suppression in vivo using HCT116 xenografts and a drug delivery system of carbonate apatite. Significant antitumor efficacy was observed in the siPTBP1 group at day 14 (Figure 5D, \(P < 0.01\)).
High PTBP1 mRNA expression correlates with poor clinicopathological variables and prognosis

The experimental samples were divided into two groups (the high-expression group with \( PTBP1 \) expression values > 0.089, \( n = 115 \), and the low-expression group, \( n = 63 \)) to investigate \( PTBP1 \) expression in association with clinicopathological variables (Table 2). The cutoff between the two groups was defined by an upper limit, including 95% of the expression values of the normal samples. Significant between-group differences were observed in venous invasion (\( P = 0.045 \), \( \chi^2 \) test), lymph node metastasis (\( P = 0.0020 \)), distant metastasis (\( P = 0.0016 \)), and Dukes stage (\( P = 0.039 \)).

Using a logistic regression model for lymph node metastasis, in univariate analysis, tumor size (\( P = 0.018 \)), histological grade (\( P = 0.024 \)), depth of tumor invasion (\( P = 0.0016 \)), lymphatic invasion (\( P < 0.0001 \)), venous invasion (\( P < 0.0001 \)), and overexpression of \( PTBP1 \) mRNA (\( P = 0.0016 \)) were significant. In multivariate analysis (Table 3), overexpression of \( PTBP1 \) mRNA was significantly associated with lymph node metastasis (\( P = 0.008 \)) as well as with lymphatic invasion (\( P = 0.0011 \)) and venous invasion (\( P = 0.043 \)). Using immunohistochemical analysis on lymph nodes, PTBP1 positive lesions were limited in lymph node with metastasis and no significant staining.
was observed in normal lymph node (Supplemental Figure S7). In addition, PTBP1 mRNA expression was positively correlated with poor survival rate after surgery (Supplemental Figure S8A; $P = 0.0138$) and poor disease-free survival after curative surgery (Supplemental Figure S8B; $P = 0.0083$). As for the CD44v8-10 expression, although poor prognosis was observed in high expression group (Supplemental Figure S9), there was no significant co-relationship between any clinico-pathological variables and CD44v8-10 expression in this series.
DISCUSSION

PTBP1, also known as HnRNP I, was first cloned and identified as playing a central role in α-tropomyosin alternative splicing (23). PTBP1 serves as a repressor of alternative splicing in mammalian cells (24-29) and contains RNA-binding domains, each of which binds to CU-rich elements (30). It is involved in polyadenylation of the pre-mRNA 3’ end (31-33) and also plays an important role in translational regulation of a subset of RNA transcription through internal ribosome entry sites (21, 34-37). As for malignancies, PTBP1 is overexpressed in glioblastoma (7, 8) and breast cancer (38), suppresses p27 expression, and contributes largely to cell proliferation (21, 39). In zebrafish, PTBP1 ablation leads to increased proliferation and cell apoptosis (40). Until now, the role of PTBP1 in CRC progression has not clearly been identified. In this study, we report that PTBP1 is preferentially overexpressed in human CRC and related to invasion and proliferation in CRC cells. Moreover, our results indicate that PTBP1 expression level is highly correlated with poor prognosis in CRC patients.

In CRC, the PKM2 isoform plays a central role in metabolism and growth (41). The important isoform switch mechanism is regulated by HnRNP proteins including PTBP1 in brain tumor cells (8). Switching the pyruvate kinase isoform to PKM1 leads to breakdown of the Warburg effect, which involves reduced lactate production and
increased oxygen consumption (41). In this study, when PTBP1 was preferentially overexpressed, the PKM2 isoform was dominant, and PTBP1 and PKM2 expression correlated in clinical CRC specimens; thus, we hypothesized that PTBP1 regulates PKM1/PKM2 splicing in CRC. In testing this hypothesis, we found that PTBP1 silencing in CRC cells leads to a shift in the prevalent PKM1 isoform.

Pursuing this finding further, we performed co-immunohistochemistry of PTBP1 and CAIX, a hypoxia marker (42), in early stage clinical specimens and found that PTBP-positive cases were associated with hypoxic lesions while PTBP1-negative cases did not contain CAIX-positive lesions. Moreover, PTBP1 was linked to the hypoxic lesions through dysregulation of PKM2 expression in CRC. This finding indicates that PTBP1 expression correlates positively with hypoxic lesions that are generally resistant to radiochemotherapy and creates conditions that promote cancer progression (43).

Although PKM2 itself promotes cell migration (44), we focused on CD44 splicing that includes a variant that induces a metastatic phenotype in tumor cells (45). In CRC, CD44 splicing variants, especially v8-10, are reported to have a strong relationship with tumor invasion (18, 19). In clinical specimens, PTBP1 expression correlated positively with CD44v8-10 mRNA expression. PTBP1 silencing in CRC cells
reduced the CD44\textsubscript{v8-10} ratio in HCT116 and DLD1, which are microsatellite-unstable cell lines that do not respond to TGF-\(\beta\)-induced EMT (46). On the other hand, HT-29 and SW480 did not respond to siPTBP1 on splicing of CD44. Based on cell invasion assay results, the invasion ratio was reduced in all analyzed cell lines. HnRNP A1 is reported to promote tumor invasion through upregulating CD44v6 in hepatocarcinoma cells (47). These findings suggest that PTBP1 might play an important role in cell invasion and that these invasive properties arise partially through CD44 splicing. Moreover, these results fit with the presence identified here of PTBP1-positive cells in the invasive front of early stage CRC specimens. Recently, a CD44 variant form was found to play an important role in promotion of intestinal cancer formation in APC (min) mice (48). In concert with these findings, we show here that PTBP1 affects cancer promotion and invasion through upregulating a CD44 variant form and that the CD44 alternative splicing to the CD44\textsubscript{v8-10} isoform regulated by PTBP1 is important, especially in microsatellite-unstable CRCs. Unfortunately, less invasive abilities with silencing PTBP1 in MSS cell lines were still elusive, further experiments were required.

Cell proliferation ability is also directly related to tumor progression. To test the effect of PTBP1 on the cell cycle and cell proliferation, we performed a proliferation assay in four cell lines. In all analyzed cases, silencing PTBP1 led to reduced
proliferation. Next, we hypothesized that the effect of PTBP1 on proliferation arose because of increased cell cycle mediators or decreased cell cycle suppressors.

Interestingly, however, although PTBP1 is reported to promote p27/Kip1 protein expression (21), silencing PTBP1 had almost no effect on p27 protein levels in CRC cells except SW480 cells. Moreover, other cell cycle mediators including Myc, Skp2, cyclin A, cyclin B, cyclin D, cyclin E, and CDC2 were overexpressed with PTBP1 suppression. Previous work has shown that in zebrafish, silencing PTBP1 leads to proliferation of intestinal epithelial cells (40), and upregulation of cell cycle mediators would be a plausible explanation for this effect. However, in the current study, actual cell proliferation was reduced in CRC cells. To clarify this discrepancy, we performed a time-lapse imaging study. In embryonic stem cells, silencing PTBP1 causes a prolonged G2/M phase (49). Here, we found that silencing PTBP1 led to an increased G2/M population, based on time-lapse tracing of HCT116 cells. With a synchronized cell cycle, PTBP1-silenced cells were entered into the cell cycle in a rapid manner; however, the percentage of cells with an active cell cycle was low. Analysis on CDK11p58, critical regulator of cell mitosis (22), partially clarified this discrepancy. Reduced expression of PTBP1 down-regulate CDK11p58, in turn, various cell cycle mediators would be up-regulated. As a result, cells go to G2/M phase in rapid manner, however no effective
mitosis was occurred, resulted in prolonged G2/M phase. In other words, several cell

mitosis was occurred, resulted in prolonged G2/M phase. In other words, several cell
cycle mediators such as cyclin A, cyclin B, cyclin C, cyclin D, cyclin E, CDC2 and
Skp2 would be up-regulated reactively to the down-regulation of CDK11p58. Although
CDK11p58 was reported to inhibit metastasis in ER-positive breast cancer, in androgen
independent prostate cancer(50), CDK11p58 was reported to have positive relationship
to tumor progression through small leucine zipper protein (sLZIP) indicate that
CDK11p58 would have distinct role on cancer progression and it would be dependent on
cancer type. Further study will clarify this discrepancy(51). We obtained similar results
in the in vivo assay. In agreement with these findings, silencing PTBP1 led to less cell
growth and an increased G2/M population. Because almost all conventional anti-cancer
drugs target cell division in the G2/M population, anti-PTBP1 therapy and conventional
chemotherapy should be a promising combination for CRC patients. In other words,
because chemoresistant cell properties play an important role in cancer stem cell theory
(52, 53), PTBP1 might affect maintenance of cancer stem cell properties. Further
studies are needed to clarify the relationship between PTBP1 expression and cancer
stem cells.

Another important finding of this study is the correlation between PTBP1
expression and clinicopathological features. Of note, high PTBP1 expression was
positively correlated with poor prognosis. In early stage cancer cases, high PTBP1 expression levels were positively correlated with lymphatic invasion, venous invasion, and number of buddings. Because the number of cases with lymph node metastasis in this series was very low (4 of 70 cases), no positive correlation was evaluable. In the advanced cancer series, high PTBP1 expression was positively correlated with lymph node metastasis, distant metastasis, and tumor stage. Multivariate analysis showed that lymphatic invasion, venous invasion, and PTBP1 expression were independently significant for lymph node metastasis. These finding also indicate that PTBP1 expression might be a useful marker for lymph node metastasis in addition to well-known markers such as lymphatic or venous invasion. Our results also suggested that PTBP1-targeting therapies could possibly lead to suppression of lymph node metastasis in patients with CRC. Development of PTBP1 inhibitors might be a novel therapeutic strategy for CRC.

In conclusion, PTBP1 is positively associated with cancer progression properties such as invasion or proliferation in CRC through upregulation of PKM2 and CD44 variants and cell cycle progression (Figure 5E). PTBP1 might serve as a marker for lymph node metastasis in CRC. As regarding CDK11p58 expression levels, there is some discrepancy. Co-relationship between PTBP1, CDK11p58, and cell behavior is
specifically depend on cell types, e.g., in ER-positive breast cancer, CDK11p58 have negative effects in cancer progression (50), in AR-independent prostate cancer, CDK11p58 have a positive relationship to the cancer progression through sLZIP (51), and, in ES cells, the protein expression and IRES activity of CDK11p58 in PTBP1/- ES cells is higher than that of wild-type ES cells, indicating that PTBP1 is involved in the repression of CDK11p58 expression through IRES-dependent translation (49). However, our data indicates that PTBP1 have a positive effect on CKD11p58 expression. Moreover, the previous report indicates that MYC might play an important role and positive effect on IRES-dependent translation (54). Further study might reveal this discrepancy.

Targeting PTBP1 would be promising strategy for radical treatment for CRC patients.
Acknowledgement

H Yamamoto conceived the project. H Takahashi, J Nishimura, Y Kagawa, Y Kano, Y Takahashi, M Hiraki, A Hamabe, and Xin Wu performed the experiments. H Takahashi, J Nishimura, M Konno, N Haraguchi, I Takemasa, T Mizushima, M Ishii, K Mimori, Y Doki, M Mori, and H Yamamoto designed the experiments and interpreted data. H Takahashi, J Nishimura and H Yamamoto co-wrote the paper.
REFERENCE


Gastroenterology. 2010;138:1406-17.
Table 1
The relationship between PTBP1 expression in the invasive front and clinicopathological variables of early CRC specimens with submucosal invasion (n = 70)

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>Negative/weakly positive (n = 31)</th>
<th>Strongly positive (n = 39)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>57.5 ± 2.08</td>
<td>60.8 ± 1.86</td>
<td>0.25</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>25/6</td>
<td>29/10</td>
<td>0.53</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30 mm/≥31 mm</td>
<td>23/7</td>
<td>31/8</td>
<td>0.78</td>
</tr>
<tr>
<td>Tumor location&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right:left</td>
<td>6/25</td>
<td>7/32</td>
<td>0.88</td>
</tr>
<tr>
<td>Gross form</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protrusion/flat</td>
<td>13/18</td>
<td>18/21</td>
<td>0.72</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/others&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20/11</td>
<td>16/23</td>
<td>0.050</td>
</tr>
<tr>
<td>Histological grade of invasive front</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/others&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22/9</td>
<td>31/8</td>
<td>0.41</td>
</tr>
<tr>
<td>Absolute infiltration distance (μm)</td>
<td>1554.8 ± 265.2</td>
<td>1943.6 ± 236.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>24/7</td>
<td>11/28</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>31/0</td>
<td>7/32</td>
<td>0.0030*</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>30/1</td>
<td>36/3</td>
<td>0.41</td>
</tr>
<tr>
<td>Number of budding</td>
<td>1.10 ± 0.43</td>
<td>2.08 ± 0.38</td>
<td>0.047*</td>
</tr>
</tbody>
</table>
SD = standard deviation; well = well-differentiated adenocarcinoma; * = significant difference ($P < 0.05$)

a, Relative to splenic flexure

b, Indicates moderately and poorly differentiated adenocarcinoma.
Table 2

The relationship between PTBP1 mRNA expression and clinicopathological variables in an advanced case series (n = 178)

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>PTBP1 mRNA expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low (n = 63)</td>
<td>high (n = 115)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>66.9 ± 1.40</td>
<td>66.7 ± 1.37</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>38/25</td>
<td>67/48</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30 mm/≥31 mm</td>
<td>12/51</td>
<td>35/80</td>
</tr>
<tr>
<td>Tumor location&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right/left</td>
<td>23/40</td>
<td>37/78</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/others&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27/36</td>
<td>35/80</td>
</tr>
<tr>
<td>Depth of tumor invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mp/ss</td>
<td>10/43</td>
<td>28/81</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>44/19</td>
<td>65/50</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>56/7</td>
<td>88/27</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>45/18</td>
<td>53/62</td>
</tr>
<tr>
<td>Distant metastasis&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent/present</td>
<td>62/1</td>
<td>98/17</td>
</tr>
<tr>
<td>Dukes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, B/C, D</td>
<td>45/18</td>
<td>54/61</td>
</tr>
</tbody>
</table>

SD = standard deviation; well = well-differentiated adenocarcinoma; mp = mucosa propria; ss = subserosa; * = significant difference (P < 0.05)
a, Relative to splenic flexure

b, Indicates moderately and poorly differentiated adenocarcinoma.

c, liver metastasis, 9 cases; lung metastasis, 2 cases; peritoneal dissemination, 4 cases;

simultaneous liver and lung metastasis, 2 cases; simultaneous liver metastasis and

peritoneal dissemination, 1 case
Table 3 Univariate and multivariate analyses for lymph node metastasis (logistic regression model)

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th></th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95% CI</td>
<td>P</td>
<td>RR</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>0.69</td>
<td>0.35–1.39</td>
<td>0.305</td>
<td>0.99</td>
<td>0.51–2.01</td>
<td>0.983</td>
</tr>
<tr>
<td>(&lt;65/≥66)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>0.99</td>
<td>0.51–2.01</td>
<td>0.983</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Male/female)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location(^a)</td>
<td>0.74</td>
<td>0.38–1.42</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Right/left)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>2.49</td>
<td>1.17–5.63</td>
<td>0.018(^*)</td>
<td>1.77</td>
<td>0.68–4.79</td>
<td>0.24</td>
</tr>
<tr>
<td>(&lt;30 mm/≥31 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td>2.14</td>
<td>1.11–4.19</td>
<td>0.024(^*)</td>
<td>1.55</td>
<td>0.70–3.48</td>
<td>0.28</td>
</tr>
<tr>
<td>(Well/others(^b))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depth of tumor invasion</td>
<td>3.53</td>
<td>1.59–8.49</td>
<td>0.0016(^*)</td>
<td>2.3</td>
<td>0.91–6.17</td>
<td>0.078</td>
</tr>
<tr>
<td>(<del>mp/ss</del>)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>5.72</td>
<td>2.94–11.6</td>
<td>&lt;0.0001(^*)</td>
<td>3.58</td>
<td>1.67–7.86</td>
<td>0.0011(^*)</td>
</tr>
<tr>
<td>(Negative/positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venous invasion</td>
<td>5.81</td>
<td>2.46–15.5</td>
<td>&lt;0.0001(^*)</td>
<td>2.77</td>
<td>1.03–8.07</td>
<td>0.043(^*)</td>
</tr>
<tr>
<td>(Negative/positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTBP1 mRNA expression</td>
<td>2.9</td>
<td>1.49–5.84</td>
<td>0.0016(^*)</td>
<td>2.89</td>
<td>1.32–6.55</td>
<td>0.008(^*)</td>
</tr>
<tr>
<td>(Low/high)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RR = relative risk; CI = confidence interval; well = well-differentiated adenocarcinoma; mp = mucosa propia; ss = subserosa; * = significant difference (P < 0.05)

\(^a\) Relative to splenic flexure

\(^b\) Indicates moderately and poorly differentiated adenocarcinoma.
FIGURE LEGENDS

Figure 1

Distribution of PKM2 and PTBP1. (A) Scheme of PKM1 and PKM2, exon 8 to exon 10. Arrows indicate primer pairs used to amplify PKM cDNA. Arrowhead indicates PstI digestion site, found only in the PKM2 isoform (CTGCAG). (B) Electrophoresis for PCR products of PKM exon 8–10 from clinical samples pre- and post-PstI digestion (normal mucosa, n = 3; colon cancer tissue, n = 7). (C) Immunofluorescence co-staining of a clinical colon cancer specimen for PKM2 and PTBP1 indicates a relationship between PKM2 and PTBP1. Scale bar: 100 μm.

Figure 2

Dominance of the PKM2 isoform is regulated by PTBP1, and a correlation between PKM2 and PTBP1 was observed in CRC specimens. (A) Downregulation of PTBP1 using siRNA causes PKM1 upregulation in CRC cell lines. (B) Immunohistochemical findings of early stage cancer specimens. Upper panel; PTBP1 positive case. In the invasive front, PTBP1 became strongly positive and the CAIX-positive lesion emerged. Lower panel; PTBP1 negative case. No CAIX-positive lesion was observed. Scale bar: 500 μm. (a) Surface of the PTBP1 positive case, almost no PTBP1 and CAIX positive
lesion was observed. (b) Middle layer of the PTBP1 positive case, PTBP1 positive lesion emerged slightly. (c) Invasive front of the PTBP1 positive case, PTBP1 positive lesions were emerged ubiquitously and partially made CAIX positive lesions. (d) Invasive front of the PTBP1 negative case, neither PTBP1 nor CAIX positive lesion was observed.

**Figure 3**

PTBP1 deregulates CD44 alternative splicing and tumor invasion. (A) qRT-PCR of clinical CRC specimens indicated correlation between CD44v8-10 and PTBP1 mRNA levels (n = 91). ***, significant difference, \( P < 0.0001 \). (B) Effect of silencing PTBP1 for CD44 alternative splicing was validated by qRT-PCR in four CRC cell lines. ***, significant difference, \( P < 0.0001 \). (C) Effect of PTBP1 silencing for cancer invasion capacity validated by invasion assay in four cell lines. Scale bar: 50 μm. ***, (D) Semi-quantified invaded cells in four cell lines (n = 3). significant difference, \( P < 0.0001 \).

**Figure 4**

Silencing PTBP1 regulates cell proliferation through a prolonged G2/M phase. (A)
Proliferation assay in HCT116, DLD1, HT-29, and SW480 cells. **, significant difference, $P < 0.001$; ***, significant difference, $P < 0.0001$. (B) Western blot of cell cycle mediators in control siRNA and silencing PTBP1 cells. β-actin was used as a control. (C) Time-lapse imaging of HCT116/Fucci cells with and without PTBP1 silencing. Quantified ratios of Green/Red cells described graphically. ***, significant difference, $P < 0.0001$, repeated-measures ANOVA.

**Figure 5**

Silencing PTBP1 resulted in attenuated colony formation and tumor progression. (A) Tracing of cell cycle after synchronization to G0/1 phase in HCT116/Fucci cells with control siRNA and siPTBP1. (B) Western blot for CDK11p58 on HCT116 and DLD1 whole cell lysate. (C) Colony formation assay. Relative cell titers were normalized to csiRNA cells. ***, significant difference, $P < 0.0001$. (D) Antitumor activity assay in vivo using carbonate apatite. Arrows indicate administration of carbonate apatite including control siRNA or siPTBP1. *, significant difference, $P < 0.01$, repeated-measures ANOVA. (E) Proposed schema of this study.
**Figure 1**

A

B

<table>
<thead>
<tr>
<th>Marker</th>
<th>normal mucosa (n = 3)</th>
<th>colon cancer tissues (n = 10)</th>
<th>Negative Control</th>
</tr>
</thead>
</table>

- **Pre PstI digestion**
  - PKM1
    - 8
    - 9
    - 11
  - PKM2
    - 8
    - 10
    - 11

- **Post PstI digestion**
  - Marker
    - M1
    - M2

C

- **Ptbp1 / Pkm2 / DAPI / Phase Contrast**
  - **Tumor Surface**
  - **Invasive Front**
  - **V**
  - **V**
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>HCT116</th>
<th>DLD1</th>
<th>HT29</th>
<th>SW480</th>
</tr>
</thead>
<tbody>
<tr>
<td>csi RNA</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>si PTBP1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti Ptbp1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti Beta-Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

PTBP1 positive case

PTBP1 negative case

PTBP1 - DAB

PTBP1

CAIX

DAPI

Merged

(a)

(b)

(c)

(d)
**Figure 3**

**A** Clinical specimens

![Graph showing CD44 mRNA levels relative to GAPDH in clinical specimens with low (n ≤ 41) and high (n ≥ 50) PTPB1 mRNA expression.]

**B** mRNA levels relative to GAPDH

![Bar graphs showing mRNA levels for HCT116, DLD1, HT29, and SW480 cells with control siRNA and siPTBP1 treatments.](image)

**C** Scale Bar = 50μm

![Images of cell cultures showing control siRNA and siPTBP1 treatments for HCT116, DLD1, HT29, and SW480 cells.](image)

**D** Number of invaded cells / HPF

![Bar graphs showing the number of invaded cells per high power field (HPF) for HCT116, DLD1, HT29, and SW480 cells with control siRNA and siPTBP1 treatments.](image)
Figure 4

A

HCT116

DLD1

HT29

SW480

Relative proliferation

Time after seeding (h)

B

HCT116 DLD1 HT29 SW480

csiRNA siPTBP1

p27

Skp2

cMyc

Fbxw7

p21

Cyclin A

Cyclin B

Cyclin D

Cyclin E

CDK2

CDK4

CDC2

CDC25A

β-Actin

C

0h 8h 16h 24h 32h 40h 48h

csiRNA

siPTBP1

Green cells / Red cells

Time (hour)

Downloaded from mct.aacrjournals.org on June 20, 2017. © 2015 American Association for Cancer Research.
Figure 5

A

Tumor volume (mm$^3$)

Days after start of treatment

B

HCT116   DLD1

csiRNA  +  -  +  -

siPTBP1  -  +  -  +

CDK11$^{p58}$

β-Actin

C

Relative colony formation to csiRNA (%)

HCT116   DLD1   HT29   SW480

csiRNA  100  100  100  100

siPTBP1  50   50   50   50

D

Tumor volume (mm$^3$)

Days after start of treatment

E

PTBP1

PKM2  CD44$^{v8-10}$  CDK11$^{p58}$

Cell Invasion  Effective Cell Mitosis

Anchorage-independent growth

Cell Cycle Mediators
Significance of polypyrimidine tract binding protein 1 expression in colorectal cancer

Hidekazu Takahashi, Junichi Nishimura, Yoshinori Kagawa, et al.

*Mol Cancer Ther* Published OnlineFirst April 22, 2015.