Significance of Polypyrimidine Tract-Binding Protein 1 Expression in Colorectal Cancer

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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 the role of PTBP1 in colorectal cancer. PTBP1 expression levels were significantly upregulated in cancerous tissues compared with corresponding normal mucosal tissues. We also observed that PTBP1 expression levels, c-MYC expression levels, and PKM2:PKM1 ratio were positively correlated in colorectal cancer specimens. Moreover, PTBP1 expression levels were positively correlated to poor prognosis and lymph node metastasis. In analyses of colorectal cancer cells using siRNA for PTBP1, we observed that PTBP1 affects 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 colorectal cancer cell lines. PTBP1 expression also affected cell proliferation. Using time-lapse imaging analysis, PTBP1 was implicated in prolonged G1–M phase in HCT116 cells. As for the mechanism of prolonged G1–M phase in HCT116 siPTBP1 cells, Western blotting revealed that PTBP1 expression level was correlated to CDK11p58 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 colorectal cancer.

Introduction

Colorectal cancer 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 colorectal cancer 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 colorectal cancer still die from disease recurrence, mainly because of liver metastasis. Therefore, further elucidation of the molecular mechanisms of colorectal cancer 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 with 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 colorectal cancers.
Here, we studied the clinicopathologic 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 colorectal cancer who underwent surgical treatment at the Kyushu University (Reppu, Japan) 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 RNA later (Takara), embedded in Tissue Tek OCT medium (Sakura), 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 histologic grade, were taken from clinical and pathologic 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 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 the ATCC. Mycoplasma testing was done also by the authors. Cells were grown in Dulbecco's modified Eagle 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 Supplementary 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 minutes, followed by 45 cycles at 95°C for 10 seconds and at 60°C for 30 seconds. 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 colorectal cancer 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 (H0005725-M01; Abnova) was used at 3 μg/mL concentration, as was anti-human carbonic anhydrase 9 (CA9) antibody (3829-1; Epitomics) 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 antimouse IgG (1:500) and Alexa Fluor 555–conjugated goat antirabbit IgG (Invitrogen; 1:1,000) 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 and colleagues (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**

The small interfering RNA (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 nontargeting siRNA) were purchased from Thermo Scientific. Cells were transfected with siRNA in 20 nmol/L 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) according to the manufacturer's protocols. Briefly, 5 × 10⁵ cells were seeded in triplicate on the Matrigel-coated membrane. After 48 hours, 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 × 10⁵ cells were seeded onto 6-well plates (BD). At 24 hours after seeding, cells were transfected with siRNAs using Lipofectamine (Invitrogen) according to the manufacturer's protocol. At 24 hours after transfection, 3 × 10⁵ cells were seeded onto a 96-well plate (BD), and relative cell numbers were determined using the Cell Counting Kit-8 (Dojindo) and the indicated time course.

**Western blotting**

Antibodies were purchased as follows: anti-PTBP1 and anti-Flk5 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).

Establishment of Fucci-expressing HCT116

A total of 5.0 × 10⁴ 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% CO₂ 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 530/30BP or 585/42BP filters, respectively.

Time-lapse imaging of Fucci-expressing HCT116

A total of 5.0 × 10⁴ 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% CO₂ 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 530/30BP or 585/42BP filters, respectively. The images were taken every other hour for 48 hours at 20 points for each sample. Raw imaging data were processed with Imaris (Bitplane) with an additional hour for 48 hours at 20 points for each sample. Raw imaging data were processed with Imaris (Bitplane) with a Gaussian filter for noise reduction. Automatic object counting was aided by manual correction to retrieve cell coordinates over time.

Colony formation assay

Cells (2 × 10⁴) were transfected with siRNAs in 6-well plates. After 48-hour culture, cells were trypsinized and 5,000 cells were reseeded into each well of CytoSelect (Cell Biolab INC). After additional 7 days of culture, cell dose was obtained, according to the manufacturer’s protocols, from absorbance (485/520 nm) using standard multiplate reader (PerkinElmer INC).

Antitumor activity assay

Seven-week-old BALB/cA nude mice were purchased from CLEA Japan. HCT116 (1.0 × 10⁵ 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 to 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: \[ V = \frac{a}{2} \times b^2 \times \frac{1}{2}, \] 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). Possible differences between groups were analyzed using the Student’s t test, \( \chi^2 \) test, Wilcoxon test, or repeated-measures ANOVA. Survival curves were obtained by the Kaplan–Meier method; comparison between curves was completed with the log-rank test. A probability level of 0.05 was chosen to indicate statistical significance.

Results

PTBP1 expression is preferentially overexpressed in clinical colorectal cancers

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; Supplementary Fig. S1A). Supplementary Fig. S1B shows the results of immunohistochemical studies of PTBP1 expression in representative clinical samples of normal mucosa (i), well-differentiated adenocarcinoma (ii), moderately differentiated adenocarcinoma (iii), and poorly differentiated adenocarcinoma (iv). 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 (Supplementary Fig. 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 colorectal cancer, we performed qRT-PCR for Myc in 64 clinical colorectal cancer specimens and identified a relationship between PTBP1 and MYC mRNA levels (Supplementary Fig. S3A; P = 0.018). In colorectal cancer cell lines, silencing Myc with siRNA reduced PTBP1 protein levels (Supplementary Fig. S3B).

PTBP1 regulates PKM isotype switch in colorectal cancer

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 to 11 was performed, followed by digestion with PstI. If exon 10 was included (PKM2), the PCR products were divided into two fragments (Fig. 1A). Normal mucosa samples had both PKM1 and PKM2 isoforms; by contrast, cancerous tissues were PKM2 dominant except in one case (Fig. 1B). Fluorescence coimmunohistochemistry for PTBP1 and PKM2 of clinical specimen revealed that while in tumor surface both of them were slightly positive (Fig. 1C, left), in invasive front both of them were ubiquitously positive (Fig. 1C, right) 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 colorectal cancer cell lines (HCT116, DLD1, HT-29, and SW480). As indicated in Fig. 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 (Fig. 2C). Next, we analyzed early-stage cancers by immunohistochemistry, dividing samples into two groups according to the grade of staining (Supplementary Fig. 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 coimunostaining for PTBP1 and CAIX in the early-stage clinical specimens. PTBP1-positive cases (Fig. 2B, left) had no CAIX-positive lesions in the surface or middle layer (Fig. 2B (a), (b)); however, focal CAIX-positive lesions were observed in the invasive front that PTBP1 was highly positive (Fig. 2B (c)); in contrast, PTBP1-negative cases had no CAIX-positive lesions even in the invasive front (Fig. 2B, right and Fig. 2B (d)).

PTBP1 expression is associated with tumor invasion through CD44 splicing

Because CD44 splicing variants in colorectal cancer, 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 colorectal cancer. 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 (Fig. 3A).

Next, we analyzed four cell lines using another primer pair (Supplementary Fig. S5A) for validation of CD44 variant expression. By gel electrophoresis (Supplementary Fig. S5B) and sequence analysis (Supplementary Fig. 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 (Fig. 3B; Supplementary Fig. S5B). In the in vitro invasion assay, silencing PTBP1 led cells to be less invasive in HCT116, DLD1, HT29, and SW480 (Fig. 3C). Calculated invaded cells ratio was also significantly reduced in silencing PTBP1 in each cell lines (Fig. 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 colorectal cancer cells, we performed a proliferation assay. When silencing PTBP1, proliferation ability was significantly reduced in HCT116, DLD1, HT29, and SW480 (Fig. 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 internal ribosome entry site (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 (Fig. 4B).

Interestingly, other various cell-cycle mediators, including cyclin A, cyclin B, cyclin C, cyclin E, CDC2, and Skp2, were increased with PTBP1 silencing (Fig. 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 (Fig. 4C, P < 0.0001). To synchronize the cell cycle, we sorted red (G1–S) cells. In the invasive front, PTBP1 become strongly positive and the CAIX-positive lesion emerged. Right, 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 emerged ubiquitously and partially made CAIX-positive lesions. d, invasive front of the PTBP1-negative case, neither PTBP1- nor CAIX-positive lesion was observed.

Significance of PTBP1 in Colorectal Cancer

Figure 2.
Dominance of the PKM2 isoform is regulated by PTBP1, and a correlation between PKM2 and PTBP1 was observed in colorectal cancer specimens. A, downregulation of PTBP1 using siRNA causes PKM1 upregulation in colorectal cancer cell lines. B, immunohistochemical findings of early-stage cancer specimens. Left, PTBP1-positive case. In the invasive front, PTBP1 become strongly positive and the CAIX-positive lesion emerged. Right, 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 emerged ubiquitously and partially made CAIX-positive lesions. d, invasive front of the PTBP1-negative case, neither PTBP1- nor CAIX-positive lesion was observed.

Protein levels were downregulated (Fig. 5B). In clinical samples, PTBP1 expression was related to the expression of CDK11p58 protein levels (Supplementary Fig. S6). In concert with these findings, PTBP1 might have an important role in progression to complete mitosis of cells though CDK11p58 regulation; in other words, silencing PTBP1 leads to prolonged G2–M phase and cell-cycle mediators might be upregulated by some feedback mechanism in these settings.

On the basis of the fact that PTBP1 expression levels have relationship to cell invasion and proliferation, we hypothesized
Table 1. The relationship between PTBP1 expression in the invasive front and clinicopathologic variables of early colorectal cancer specimens with submucosal invasion (n = 70).

<table>
<thead>
<tr>
<th>Clinicopathologic 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>Male/female 25/6</td>
<td>29/10</td>
<td>0.53</td>
</tr>
<tr>
<td>Tumor diameter (&lt;30 mm/≥31 mm)</td>
<td>23/7</td>
<td>31/8</td>
<td>0.78</td>
</tr>
<tr>
<td>Tumor location*</td>
<td>Right/Left 6/25</td>
<td>7/32</td>
<td>0.88</td>
</tr>
<tr>
<td>Gross form</td>
<td>Protrusion/Flat 13/18</td>
<td>18/21</td>
<td>0.72</td>
</tr>
<tr>
<td>Histologic grade Well/others*</td>
<td>20/7</td>
<td>16/23</td>
<td>0.05</td>
</tr>
<tr>
<td>Histologic grade of invasive front Well/others*</td>
<td>22/9</td>
<td>31/8</td>
<td>0.41</td>
</tr>
<tr>
<td>Absolute infiltration distance (μm)</td>
<td>1,554.8 ± 265.2</td>
<td>1,943.6 ± 236.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>Absent/present 24/7</td>
<td>11/28</td>
<td>&lt;0.0003*</td>
</tr>
<tr>
<td>Venous invasion</td>
<td>Absent/present 31/0</td>
<td>7/32</td>
<td>0.0030*</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>Absent/present 30/1</td>
<td>36/3</td>
<td>0.41</td>
</tr>
<tr>
<td>Number of budding</td>
<td>110 ± 0.43</td>
<td>2.08 ± 0.38</td>
<td>0.047*</td>
</tr>
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Abbreviation: well, well-differentiated adenocarcinoma.
*aModerately and poorly differentiated adenocarcinoma.
*bSignificant difference (P < 0.05).

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).

In colorectal cancer, 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 colorectal cancer specimens; thus, we hypothesized that PTBP1 regulates PKM1/PKM2 splicing in colorectal cancer. In testing this hypothesis, we found that PTBP1 silencing in colorectal cancer cells leads to a shift in the prevalent PKM1 isoform.

Pursuing this finding further, we performed immunohistochemistry of PTBP1 and CAIX, a hypoxia marker (42), in early-stage clinical specimens and found that PTBP1-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 colorectal cancer. 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 colorectal cancer, CD44 splicing variants, especially v8-10, are reported to have a strong relationship with tumor invasion (18, 19).

In colorectal cancer, PTBP1 mRNA expression correlates with poor clinicopathologic 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 clinicopathologic 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, χ^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), histologic 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 (Supplementary Fig. S7). In addition, PTBP1 mRNA expression was positively correlated with poor survival rate after surgery (Supplementary Fig. S8A; P = 0.0138) and poor disease-free survival after curative surgery (Supplementary Fig. S8B; P = 0.0083). As for the CD44_v8-10 expression, although poor prognosis was observed in high expression group (Supplementary Fig. S9), there was no significant correlation between any clinicopathologic variables and CD44_v8-10 expression in this series.
specimens, PTBP1 expression correlated positively with CD44v8-10 mRNA expression. PTBP1 silencing in colorectal cancer cells reduced the CD44v8-10 ratio in HCT116 and DLD1, which are microsatellite-unstable cell lines that do not respond to TGFβ-induced EMT (46). On the other hand, HT-29 and SW480 did not respond to siPTBP1 on splicing of CD44. On the basis of 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

**Figure 3.** PTBP1 deregulates CD44 alternative splicing and tumor invasion. A, qRT-PCR of clinical colorectal cancer specimens indicated correlation between CD44v8-10 and PTBP1 mRNA levels (n = 9). ***, significant difference, P < 0.0001. B, effect of silencing PTBP1 for CD44 alternative splicing was validated by qRT-PCR in four colorectal cancer cell lines. ***, significant difference, P < 0.0001; n.s., not significant. C, effect of PTBP1 silencing for cancer invasion capacity validated by invasion assay in four cell lines. Scale bar, 50 μm. D, semiquantified invaded cells in four cell lines (n = 3). ***, significant difference, P < 0.0001.
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 colorectal cancer 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 CD44v8-10 isoform regulated by PTBP1 is important, especially in microsatellite-unstable colorectal cancer. 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 increased 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 colorectal cancer 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 this study, actual cell proliferation was reduced in colorectal cancer 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 downregulate CDK11p58, in turn, various cell-cycle mediators would be upregulated. 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-cycle mediators, such as cyclin A, cyclin B, cyclin C, cyclin D, cyclin E, CDC2, and Skp2, would be upregulated reactively to the downregulation 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
Table 2. The relationship between PTBP1 mRNA expression and clinicopathologic variables in an advanced case series (n = 178).

<table>
<thead>
<tr>
<th>Clinicopathologic variables</th>
<th>PTBP1 mRNA expression</th>
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<tbody>
<tr>
<td></td>
<td>Low (n = 63)</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>66.9 ± 1.40</td>
</tr>
<tr>
<td>Sex</td>
<td>38/25</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;30 mm/≥31 mm</td>
</tr>
<tr>
<td>Tumor location (right/left)</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>Right/left</td>
</tr>
<tr>
<td>Well/otherb</td>
<td></td>
</tr>
<tr>
<td>Depth of tumor invasion</td>
<td>mp/ss</td>
</tr>
<tr>
<td>Venous invasion</td>
<td>Absent/present</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>Absent/present</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>Absent/present</td>
</tr>
<tr>
<td>Dukes A, B/C, D</td>
<td>45/18</td>
</tr>
</tbody>
</table>

Abbreviations: mp, mucosa propria; ss, subserosa; well, well-differentiated adenocarcinoma.
aRelative to splenic flexure.
bModerately and poorly differentiated adenocarcinoma.
cLiver 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.
dSignificant difference (P < 0.05).

Table 3. Univariate and multivariate analyses for lymph node metastasis (logistic regression model).

<table>
<thead>
<tr>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Age (&lt;65/≥66)</td>
<td>0.69 (0.35-1.39)</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>0.99 (0.51-2.01)</td>
</tr>
<tr>
<td>Tumor location (right/left)</td>
<td>0.74 (0.38-1.42)</td>
</tr>
<tr>
<td>Tumor size (&lt;30 mm/≥31 mm)</td>
<td>2.49 (1.17-5.63)</td>
</tr>
<tr>
<td>Histologic grade (well/otherb)</td>
<td>2.14 (1.00-4.99)</td>
</tr>
<tr>
<td>Depth of tumor invasion (&lt;mp/ss&gt;–)</td>
<td>3.55 (1.59-8.49)</td>
</tr>
<tr>
<td>Venous invasion (negative/positive)</td>
<td>5.72 (2.94-11.6)</td>
</tr>
<tr>
<td>PTBP1 mRNA expression (low/high)</td>
<td>5.81 (2.46-15.5)</td>
</tr>
<tr>
<td>2.9 (1.45-5.84)</td>
<td>0.001c</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; mp, mucosa propria; RR, relative risk; ss, subserosa; well, well-differentiated adenocarcinoma.
aRelative to splenic flexure.
bModerately and poorly differentiated adenocarcinoma.
cSignificant difference (P < 0.05).
Disclosure of Potential Conflicts of Interest

H. Ishii reports receiving commercial research support from Taiho Co. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: H. Takahashi, J. Nishimura, T. Mizushima, H. Ishii, M. Mori

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Takahashi, T. Mizushima, M. Ishii, H. Ishii

Study supervision: H. Takahashi, H. Ishii, Y. Doki, M. Mori, H. Yamamoto

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