Expression of Coding Region Determinant-Binding Protein (CRD-BP) in Colorectal Cancer and Its Influence on Target Gene Expression and Clinical Characteristics

Yasuto Tomita 1,2,*, Takahiro Domoto 2, Yoshimichi Ueda 3, Toshinari Minamoto 2, Takeo Kosaka 1

Abstract: Purpose: Our earlier study showed that coding region determinant-binding protein (CRD-BP), an RNA trans-factor, stabilized the mRNA of β-transducin repeat-containing protein 1 (β-TrCP1), c-myc and insulin-like growth factor (IGF)-II, thereby promoting colorectal cancer (CRC) cells proliferation. Here we examined CRD-BP expression in human CRC tumors, its influence on the expression of target genes (β-TrCP1, c-myc, IGF-II) and its association with clinicopathological factors in CRC patients, including survival, in order to determine whether it could be a useful molecular marker in CRC. Methods: cDNA was prepared from paired tumor and normal mucosa tissue samples obtained from 42 CRC patients undergoing operation. Relative expression levels for CRD-BP, β-TrCP1, c-myc and IGF-II mRNA were measured using quantitative reverse transcription-PCR and the ΔΔCt method. Expression and subcellular localization of CRD-BP and β-catenin in tumors were examined by immunohistochemistry. Results: Expression of CRD-BP mRNA was greater in tumors with β-catenin nuclear accumulation (p=0.05). Increased expression of CRD-BP, β-TrCP1, c-myc and IGF-II was found in 66.7%, 71.4%, 61.9% and 52.4%, respectively, of tumors from CRC patients relative to matching normal mucosa. The expression of CRD-BP was associated with increased expression of β-TrCP1 (p<0.01 in chi-square [χ²] test, <0.001 in Spearman’s rank-correlation coefficient [Rs] test), c-myc (p=0.013 [χ² test], 0.025 [Rs test]) and IGF-II (p=0.382 [χ² test], 0.026 [Rs test]). Increased CRD-BP expression was significantly associated with younger patient age, but not with other clinicopathological factors. CRC patients exhibiting CRD-BP tumor expression showed trends for worse 5-year recurrence-free and overall survival rates compared to those without expression. Conclusion: These findings suggest that CRD-BP induces β-TrCP1 and c-myc expression in response to β-catenin activation, thus leading to tumor proliferation. Tumor CRD-BP expression may also be a prognostic marker for worse outcome in CRC patients.

Key Words: colorectal cancer, β-catenin, CRD-BP, β-TrCP1, c-myc

Abbreviations
A: adventitia; AI: adventitia invasion; CRC: colorectal cancer; CRD-BP: coding region determinant-binding protein; ΔΔCt: comparative Ct; EGFR: epidermal growth factor receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IGF-II: insulin-like growth factor-II; IGF-IIBP-1: IGF-II mRNA-binding protein-1; β-TrCP1: β-transducin repeat-containing protein 1; IHC: immunohistochemistry; I-Ba: NF-κB inhibitor α; KH: K homology; M: membranous expression; MP: muscularis propria layer; NA: nuclear accumulation; NF-κB: nuclear factor of κ light polypeptide gene enhancer in B-cell; OS: overall survival; PBS: phosphate-buffered saline; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; RFS: recurrence-free survival; RRM: RNA recognition motifs; Rs: Spearman’s rank correlation coefficient; SE: serosal exposure; SI: serosal invasion; SS: subserosal layer; Tcf: T-cell factor; β-TrCP1: β-transducin repeats-containing protein 1; VEGFR: vascular endothelial growth factor; WAP: whey acidic protein; ZBP1: zipcode-binding protein 1

Introduction
Although substantial progress has been made in the diagnosis and treatment of colorectal cancer (CRC), the number of patients with advanced and recurrent CRC is still increasing at a steady rate (1). Prognosis has been improved somewhat with the use of molecular-targeted agents (antibodies) that inhibit epidermal growth factor receptor (EGFR) or vascular...
endothelial growth factor (VEGF) (2), although a high
degree efficacy has yet to be achieved using these
therapeutic antibodies. It is therefore crucial to
ecluciate the underlying molecular pathology of CRC
in order to develop more effective targeted treatments
for this disease.

Activation of Wnt signaling is one of the critical
pathways in the carcinogenesis of CRC (3). The
aberrant mechanisms in the Wnt pathway have been
analyzed in CRC, with β-catenin activation at the
tumor-host interface shown to be a critical step in
tumor progression (4). The dual functions (cell
adhesion and signal transduction) of β-catenin, a key
mediator in the Wnt signaling pathway, are dictated
by its stability and subcellular localization (5). Studies
of transcriptional targets for β-catenin following its
translocation to the nucleus and activation are aimed
at identifying possible diagnostic markers and
therapeutic targets for cancer (6).

Various transcriptional targets of the β-catenin and
T-cell factor (Tcf) complex have been shown to
contribute to the development and progression of
cancer. One such example is coding region
determinant-binding protein (CRD-BP), previously
identified by our group as a transcriptional target of
the β-catenin/Tcf complex in CRC cells (7). CRD-BP
is an RNA trans-factor composed of 577 amino acids
and containing two RNA recognition motifs (RRM),
four K homology (KH) domains and an arginine-
glycine-glycine (RGG) box. The RNA-binding domain
of CRD-BP is conserved in a family of proteins that
includes zipcode-binding protein 1 (ZBP1) and
insulin-like growth factor-II mRNA-binding protein-1
(IGF-IIBP-1 or IMP-1), indicating homology of these
molecules with CRD-BP (8, 9). CRD-BP is expressed
in early embryonic cells, but its expression level is
extremely low or absent in normal adult tissues (10).
CRD-BP have been implicated in the creation of
cellular polarity during the early stages of
development, in developing neurons and in motile
cells, by promoting the localization of their RNA
targets to subcellular compartments (11). However,
CRD-BP expression is increased in tumor cells from
breast cancer (12), CRC (13), ovarian cancer (14) and
melanoma (15). Previous studies reported that CRD-
BP causes post-transcriptional stabilization of the
mRNA for molecules involved in carcinogenesis.
These include β-transducin repeat-containing protein 1
(β-TrCP1), which is an E3 ubiquitin ligase for
β-catenin (16), as well as nuclear factor of κ light
polypeptide gene enhancer in B-cell (NF-κB) inhibitor
α (IκBα), c-myc and IGF-II (9). These findings
suggest that CRD-BP is a functional oncofetal protein.

Previous studies have investigated CRD-BP
expression in tumor tissues and its association with
clinical tumor characteristics, tumor stage and
prognosis in patients with CRC or ovarian cancer (11-
13). However, to our knowledge there have been no
integrative studies that correlate CRD-BP expression
with β-catenin activation or with the expression of
tumor-promoting molecules that are stabilized by
CRD-BP. We previously reported that CRD-BP
stabilizes the transcripts for β-TrCP1, c-myc and IGF-
II in response to β-catenin signaling in human CRC
cells, thus implicating it in multiple pathways (Wnt/
β-catenin, NF-κB, c-myc, IGF-II, hedgehog) of tumor
cell proliferation (7, 17).

Aim

The aim of this study was to investigate tumor
expression of CRD-BP in clinical CRC, together with
that of its target molecules for mRNA stabilization.
CRD-BP expression was also correlated with
clinicopathological factors and with the postoperative
survival of CRC patients in order to determine
whether it could be a useful molecular marker in this
cancer type.

Materials and Methods

Patients and Specimens

This study investigated 42 CRC patients who
underwent surgery at the Cancer Research Institute
Hospital of Kanazawa University between 1998 and
2004 and who were enrolled in the Human
Gastrointestinal Cancer Tissue Bank Registry at the
Cancer Research Institute of Kanazawa University.
Initially, a total of 74 patients were subjected. Of
them, clinicopathological factors, postoperative
survival periods and quantitative reverse-transcription
(qRT)-PCR analysis were unavailable in 7, 11 and 14
patients, respectively. Eventually 42 CRC patients
were eligible for this study. Informed consent was
obtained from all patients after they received a
thorough explanation preoperatively regarding
enrollment in the tissue bank registry and the use of
specimens for research. Samples of paired tumor
tissue and adjacent normal mucosa were collected
from the fresh surgical specimen of each patient and
stored at −80 °C for preparation of total RNA.
Immediately after collection, the surgical specimens
were fixed in 10% buffered formalin for 48 hours and
embedded in paraffin. Subsequently, paraffin sections
of representative tissue that reflected the pathological
characteristics of the tumor and included normal
mucosa were examined by immunohistochemistry.
Analysis of Patient Characteristics and Survival

The CRC patients included 30 men and 12 women with a median age of 71.5 years (range: 33 to 88). The clinical and histopathologic characteristics for each patient were recorded according to the second English edition of the Japanese Classification of Colorectal Carcinoma (18). The site of primary tumor origin was colon (n=32) and rectum (n=10). The major histological type of tumor was differentiated adenocarcinoma (n=38). Depth of tumor invasion was classified as: stage I (n=1), stage II (n=23), stage III (n=17), and stage IV (n=1) (Table 1). Patients were followed from the day of primary surgery until death, or in the case of surviving patients until they were lost to follow-up or when the outcome survey was discontinued. Follow-up was discontinued when patients could no longer be contacted due to death from other disease or unknown clinical course.

Table 1. Characteristics of patients with colorectal cancer.

<table>
<thead>
<tr>
<th>Clinicopathological factor</th>
<th>Number of cases</th>
<th>Incidence (%)</th>
</tr>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>30</td>
<td>71</td>
</tr>
<tr>
<td>female</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
</tr>
<tr>
<td>median</td>
<td>71.5</td>
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<td>colon</td>
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<td>76</td>
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<tr>
<td>rectum</td>
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<td>24</td>
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<tr>
<td>Tumor histology</td>
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<td>Mucinous type</td>
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<td>Depth of tumor invasion</td>
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<tr>
<td>T1 (SM)</td>
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<tr>
<td>T2 (MP)</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>T3 (SS, A)</td>
<td>28</td>
<td>66</td>
</tr>
<tr>
<td>T4 (SE, SI/Al)</td>
<td>11</td>
<td>26</td>
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</tr>
<tr>
<td>Stage IV</td>
<td>1</td>
<td>3</td>
</tr>
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</table>

1Japanese Society for Cancer of the Colon and Rectum (JSCCR), Japanese classification of colorectal carcinoma, second English edition (18). Abbreviations: SM, submucosal layer; MP, muscularis propria layer; SS, subserosal layer; A, adventitia; SE, serosal exposure; SI, serosal invasion; AI, adventitia invasion.

cancer-related deaths (excludes death due to other diseases) up to September 2007 when the outcome survey was discontinued. Follow-up was discontinued when patients could no longer be contacted due to death from other disease or unknown clinical course.

Extraction of Total RNA from Tissue Specimens

Frozen tissues were homogenized in 1 mL of ISOGEN II® (Nippon Gene Material, Toyama, Japan), mixed with 0.4 mL of RNase-free water and then incubated at room temperature for 15 minutes. Subsequently, the samples were centrifuged (12 K×g, 15 minutes) and 1 mL of supernatant was collected. After adding 1 mL of isopropanol to the supernatant, the samples were mixed by inversion and incubated at room temperature for 10 minutes. The samples were centrifuged again (12 K×g, 10 minutes) and 0.5 mL of 75% ethanol was added to the precipitate. The samples were then centrifuged again (8 K×g, 3 minutes) and the precipitates were dissolved in RNase-free water to make a 200 μg/mL total RNA solution. They were stored at −80 ºC until used to synthesize complementary (c)DNA.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

An aliquot of 10 μL total RNA solution (200 μg/ mL) was mixed with 10 μL RT-PCR-Mix (RT buffer, 4 μL; 0.1 M dithiothreitol, 2 μL; 10 mM dNTP, 2 μL; 3 mg/mL bovine serum albumin, 0.5 μL; random primer, 0.25 μL; dH 2O, 1.25 μL for one reaction) and reverse-transcribed by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, MA, USA) at 45 ºC for 60 minutes using the PTC200 Thermal Cycler Dual 48 well (MJ Research, New Boston, USA) to synthesize cDNA. These cDNA samples were stored at −40 ºC until further use. For analysis of the mRNA expression of each target molecule, qRT-PCR was performed using 2 μL of cDNA and SYBR® Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) with SYBR Green I as a fluorescent reference dye. Primer sequences for qRT-PCR analysis of each target molecule are shown in Table 2. Using Stratagene Mx 3000X (Agilent Technologies, Santa Clara, USA), following an initial reaction (95 ºC, 30 seconds), 40 cycles of a denaturation reaction (95 ºC, 30 seconds) and an annealing/elongation reaction (60 ºC, 30 seconds) were performed to amplify the respective genes. The expression level of each gene (CRD-BP, β-TrCP1, c-myc, IGF-II) was determined by calculating the ΔCt value, which is the inter-specimen difference between the qRT-PCR Ct value of the respective target genes...
The chi-square ($\chi^2$) test was used to analyze the mRNA expression of each molecule as determined by qRT-PCR and IHC in CRC specimens. Mann-Whitney U test was used to compare the expression of CRD-BP mRNA expression in the tumors with different subcellular localization (M or NA) of CRD-BP. Spearman’s rank correlation coefficient ($R_s$) was used to compare the expression level of each molecule between tumor and normal tissues in the same CRC patient, as well as to correlate the mRNA expression levels of each molecule in tumor tissues.

### Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded 4 μm-thick tissue sections were immunostained for CRD-BP as described in our previous studies (21, 22). Briefly, tissue sections were deparaffinized and autoclaved (121 °C, 15 minutes) in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. After washing with phosphate-buffered saline (PBS, pH 7.4), the sections were incubated in 1% hydrogen peroxide for 30 minutes at room temperature to inactivate endogenous peroxidase and then incubated in PBS containing 10% normal goat serum (Dako, California, USA) for 60 minutes at room temperature to block non-specific antigen-antibody reactions. Subsequently, the sections were incubated with anti-CRD-BP antibody (1:200, Anti-IGF2BP1 antibody ab100999; Abcam plc, Cambridge, UK) overnight at 4 °C. After washing three times with PBS, the sections were incubated with anti-rabbit IgG secondary antibody (1:100, Dako) for 30 minutes at room temperature, washed again with PBS, and then incubated with Streptavidin-HRP (1:300, Dako) for 30 minutes at room temperature. Immunoreactants were detected by diaminobenzidine (Dako) followed by nuclear staining with hematoxylin. The sections were dehydrated and subsequently sealed with Entellan mounting medium. The CRC tissue specimen from a patient who exhibited CRD-BP protein expression in our previous immunoblot analysis (7) was used as a positive control for IHC. CRD-BP expression was classified as positive or negative, where positive expression was defined as staining with greater intensity in the nucleus compared to the cytoplasm of tumor cells, whereas negative expression was defined as the absence of staining, or equal staining between the nucleus and cytoplasm.

Results for β-catenin immunostaining were obtained from our previous studies (21, 22) that investigated specimens from the same patients as the current study. β-catenin expression and subcellular localization in tumor cells was classified into membranous expression (M) and nuclear accumulation (NA), as described previously (20, 21).

### Statistical Analysis

StatFlex ver. 6.0® (Artech Co., Ltd., Osaka, Japan) was used for statistical analysis of clinicopathological factors, postoperative survival and the expression of molecules of interest (CRD-BP, β-TrCP1, c-myc, IGF-II) as determined by qRT-PCR and IHC in CRC specimens. Mann-Whitney U test was used to compare the levels of CRD-BP mRNA expression in the tumors with different subcellular localization (M or NA) of β-catenin. Spearman’s rank correlation coefficient ($R_s$) was used to compare the expression level of each molecule between tumor and normal tissues in the same CRC patient, as well as to correlate the mRNA expression levels of each molecule in tumor tissues.

### Table 2. Sequences of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRD-BP</td>
<td>5'-CTGAGATCCTGGCCCATATAA'3'</td>
<td>5'-AAGGTCTGCACAGGAGAA'3'</td>
</tr>
<tr>
<td>β-TrCP1</td>
<td>5'-ACTGCGAAGTGAAACAGC'3'</td>
<td>5'-CATCATCTGGAAGACGACG'3'</td>
</tr>
<tr>
<td>c-myc</td>
<td>5'-GCAGCTGCTTACACGCTGA3'</td>
<td>5'-GCAGCTGCTTACACGCTGA3'</td>
</tr>
<tr>
<td>IGF-II</td>
<td>5'-AACAAATTGCGAAAATTAAGG3'</td>
<td>5'-CCAGTTTACCTGAAATCC3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GACCGTCAAGGCTGAGAC5'</td>
<td>5'-ATGGTGAAGACGCCAGT'5'</td>
</tr>
</tbody>
</table>

Abbreviations: CRD-BP, coding region determinant-binding protein; β-TrCP1, β-transducin repeats-containing protein 1; IGF-II, insulin-like growth factor-II; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

CRD-BP influences its target genes expression and clinical characteristics in CRC.

and the Ct value of the internal standard gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was amplified under the same conditions. To ensure the quality of mRNA for expression analysis, the cDNA samples from 14 of 74 cases showing more than 35 of qRT-PCR Ct value in GAPDH were excluded as described above. The comparative Ct (ΔΔCt) method (19) was used to determine differences in gene expression between paired tumor and normal mucosa tissues from the same patient by reference to the ΔCt value of each target gene in the normal mucosa tissue as a calibrator. qRT-PCR analysis of each gene was conducted in duplicate and the mean values were used for analysis. The expression of each target molecule in tumor tissue was classified into positive or negative, where positive expression was defined as staining with greater intensity in the nucleus compared to the cytoplasm of tumor cells, whereas negative expression was defined as the absence of staining, or equal staining between the nucleus and cytoplasm.

Results for β-catenin immunostaining were obtained from our previous studies (21, 22) that investigated specimens from the same patients as the current study. β-catenin expression and subcellular localization in tumor cells was classified into membranous expression (M) and nuclear accumulation (NA), as described previously (20, 21).

### Statistical Analysis

StatFlex ver. 6.0® (Artech Co., Ltd., Osaka, Japan) was used for statistical analysis of clinicopathological factors, postoperative survival and the expression of molecules of interest (CRD-BP, β-TrCP1, c-myc, IGF-II) as determined by qRT-PCR and IHC in CRC specimens. Mann-Whitney U test was used to compare the levels of CRD-BP mRNA expression in the tumors with different subcellular localization (M or NA) of β-catenin. Spearman’s rank correlation coefficient ($R_s$) was used to compare the expression level of each molecule between tumor and normal tissues in the same CRC patient, as well as to correlate the mRNA expression levels of each molecule in tumor tissues.

The chi-square ($\chi^2$) test was used to analyze the mRNA expression of each molecule as determined by the ΔΔCt method, to evaluate expression of CRD-BP mRNA expression of each molecule as determined by qRT-PCR and IHC in CRC specimens. Mann-Whitney U test was used to compare the expression level of each molecule between tumor and normal tissues in the same CRC patient, as well as to correlate the mRNA expression levels of each molecule in tumor tissues. The log-rank test was used for statistical analysis of clinicopathological factors, postoperative survival and the expression of molecules of interest (CRD-BP, β-TrCP1, c-myc, IGF-II) as determined by qRT-PCR and IHC in CRC specimens. Mann-Whitney U test was used to compare the levels of CRD-BP mRNA expression in the tumors with different subcellular localization (M or NA) of β-catenin. Spearman’s rank correlation coefficient ($R_s$) was used to compare the expression level of each molecule between tumor and normal tissues in the same CRC patient, as well as to correlate the mRNA expression levels of each molecule in tumor tissues.

The log-rank test was used to compare tumor CRD-BP mRNA expression and patient outcome in terms of overall survival (OS) and recurrence-free survival (RFS). A P value of < 0.05 was considered statistically significant for all tests.
Results

CRD-BP mRNA and Protein Expression in CRC Tumors

Comparison of CRD-BP mRNA expression between tumor and normal tissues by qRT-PCR and \( \Delta \Delta C_t \) method revealed that two thirds (28/42, 66.7%) of patients showed expression of CRD-BP in their tumor (Figure 1). For IHC analysis of CRD-BP expression, tumor tissue from a CRC patient with elevated CRD-BP expression as determined previously by Western blotting (7) was used as a positive control. In this tumor, nuclear localization of CRD-BP was prominently observed in the tumor cells, but not in the non-neoplastic crypt epithelial cells, whereas its cytoplasmic expression was found in both cell types (Figure 2). Following IHC of tumors from all CRC patients, preferential nuclear expression of CRD-BP was more intensely found in the tumor cells than adjacent non-neoplastic crypt cells in 33 cases (78.5%). Statistical analysis by \( \chi^2 \) test showed no significant correlation between the expression of CRD-BP mRNA and protein in the tumors (Table 3).

\[ \text{β-catenin and CRD-BP Expression in Tumor Tissues} \]

Based on our previous finding that \( \beta \)-catenin enhanced the transcription of CRD-BP in CRC cells (7), we investigated whether the expression and subcellular localization of \( \beta \)-catenin was correlated with CRD-BP expression in tumor cells. IHC investigation revealed nuclear accumulation of \( \beta \)-catenin in tumors from 21 patients (50%) and membranous expression in 21 patients (50%) (Table 3). Expression of CRD-BP mRNA was significantly greater in tumors with

\[ \text{Figure 1. Expression of CRD-BP mRNA in CRC tumors. CRD-BP mRNA expression was quantified in the tumor relative to that in the paired normal mucosa in the same patient using qRT-PCR and } \Delta \Delta C_t \text{ methods and shown as water-fall plots.} \]

\[ \text{Figure 2. Representative immunohistochemical expression of CRD-BP in the normal mucosa (a) and colon cancer (b, c) of a patient investigated in our previous study (7).} \]
CRD-BP influences its target genes expression and clinical characteristics in CRC.

β-catenin nuclear accumulation compared to those with membranous expression (Figure 3, Table 4).

**β-TrCP1, c-myc and IGF-II mRNA Expression in Tumor Tissues**

The mRNA expression of β-TrCP1, c-myc and IGF-II mRNA, which are stabilized by CRD-BP after transcription, was determined in tumor tissues using qRT-PCR and ΔΔCt methodology similar to that used for CRD-BP. This analysis found that β-TrCP1 expression was positive in 30 patients (71.4%) and negative in 12 patients (28.6%) (Figure 4a), c-myc expression was positive in 26 patients (61.9%) and negative in 16 patients (38.1%) (Figure 4b), and IGF-II expression was positive in 22 patients (52.4%) and negative in 20 patients (47.6%) (Figure 4c).

**Correlation of CRD-BP mRNA Expression with β-TrCP1, c-myc and IGF-II mRNA Expression in Tumor Tissues**

The expression level of CRD-BP and of its target molecules for RNA stabilization in the tumor tissues was measured using the qRT-PCR and ΔΔCt methods and compared using Spearman’s rank correlation coefficient (Rs). This analysis showed positive correlations between CRD-BP expression and the expression of its target molecules: Rs=0.605 for β-TrCP1 (P<0.001), Rs=0.346 for c-myc (P=0.03), and Rs=0.343 for IGF-II (P=0.03) (Figure 4d-f). Statistical comparison by χ² test showed that the expression of CRD-BP was significantly correlated with β-TrCP1 (p<0.01) and c-myc (p=0.01), but not with IGF-II (Table 3).

<table>
<thead>
<tr>
<th>CRD-BP mRNA</th>
<th>Membranous expression</th>
<th>Nuclear accumulation</th>
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<tbody>
<tr>
<td>Number of cases</td>
<td>Number of cases</td>
<td>p-value*</td>
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<tr>
<td>CRD-BP mRNA</td>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>(+) 6</td>
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<td>(+) 15</td>
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<tr>
<td>(+) 33</td>
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<tr>
<td>β-TrCP1 mRNA</td>
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<td>8</td>
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<tr>
<td>(+) 30</td>
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<td>c-myc mRNA</td>
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<td>9</td>
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<tr>
<td>(+) 26</td>
<td>5</td>
<td>0.38</td>
</tr>
<tr>
<td>IGF-II mRNA</td>
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<td>(+) 22</td>
<td>6</td>
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</table>

*χ² test
Abbreviations: CRD-BP, coding region determinant-binding protein; β-TrCP1, β-transducin repeats-containing protein; IGF-II, insulin-like growth factor-II

Table 3. Comparison of CRD-BP, β-TrCP1, c-myc and IGF-II expression in colorectal cancer cases.

<table>
<thead>
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<th>CRD-BP</th>
<th>β-TrCP1</th>
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<th>IGF-II</th>
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<tr>
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<td>(-) 9</td>
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<td>(+) 33</td>
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<tr>
<td>Number of cases</td>
<td>(%)</td>
<td>(%)</td>
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*χ² test
Abbreviations: CRD-BP, coding region determinant-binding protein; β-TrCP1, β-transducin repeats-containing protein; IGF-II, insulin-like growth factor-II

Table 4. Comparison of the expression of β-catenin and CRD-BP in colorectal cancer.
Figure 4. Comparison of CRD-BP expression with β-TrCP1, c-myc and IGF-II expression in CRC tumors. Relative mRNA expression of β-TrCP1 (a), c-myc (b) and IGF-II (c) in the tumors was determined using qRT-PCR and ΔΔCt methods. Each column represents data from one patient. Open and closed columns indicate tumors with or without CRD-BP mRNA expression, respectively. (d-f) The CRD-BP mRNA expression level was compared with β-TrCP1 (d), c-myc (e) and IGF-II (f) mRNA expression levels by scatter plots and calculation of the Spearman’s rank correlation coefficient (Rs).

Table 5. Comparison of tumor CRD-BP mRNA expression and clinical and histopathological characteristics of colorectal cancer.

<table>
<thead>
<tr>
<th>CRD-BP mRNA</th>
<th>(-)</th>
<th>(+)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>≥65</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>&lt;65</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>sex</td>
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<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Histological type</td>
<td>Differentiated type</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Mucinous type</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Depth of tumor invasion**</td>
<td>T1-T2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>T3-T4</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>(-)</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Lymphocytic infiltration</td>
<td>(-)</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Venous infiltration</td>
<td>(-)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Stage**</td>
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<td>8</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>III/IV</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

*p-value* *χ² test
T1: SM; T2: MP; T3: SS/A; T4: SE, SI/A
Abbreviations: CRD-BP, coding region determinant-binding protein; SM, submucosal layer; MP, muscularis propria layer; SS, subserosal layer; A, adventitia; SE, serosal exposure; SI, serosal invasion; AI, adventitia invasion
Correlation of Tumor CRD-BP mRNA Expression with Clinicalopathologic Factors and Postoperative Survival in CRC Patients

Tumor CRD-BP mRNA expression and the clinicopathologic characteristics of CRC patients were statistically compared by using $\chi^2$ test (Table 5). CRD-BP expression was observed more frequently in tumors from younger (< 65-years) patients ($p=0.03$). No significant correlations were found between CRD-BP expression and the other characteristics including sex, tumor site, histological type, depth of tumor invasion, lymphatic infiltration, venous infiltration, lymph node metastasis and stage (Table 5). The association between CRD-BP mRNA expression and patient outcome was analyzed by the Kaplan-Meier method and log-rank test. Although no significant differences were observed between patients with or without CRD-BP expression in terms of 5-year OS (76.5% vs. 90.0%, respectively; $p=0.35$) and 5-year RFS (74.6% vs. 85.7%, respectively; $p=0.48$), the former patient group showed a trend for worse prognosis (Figure 5a, b).

Discussion

The present study found that CRD-BP was expressed in the tumors of two-thirds of CRC patients. The expression level of CRD-BP mRNA was higher in tumors that also exhibited nuclear accumulation of $\beta$-catenin, indicating its activation in tumor cells. This finding is consistent with our earlier pilot study that demonstrated a biochemical association between activated $\beta$-catenin and CRD-BP expression in colon cancer cells and in tumors from a small number of CRC patients (7). In the present study, CRC tumors with CRD-BP expression exhibited significantly higher expression of $\beta$-TrCP1 and c-myc, consistent with the role of CRD-BP as an RNA trans-factor. $\beta$-TrCP1 and c-myc are known targets for mRNA stabilization by CRD-BP. This is the first study to compare the expression of CRD-BP with its target molecules in primary tumor specimens and to demonstrate significant association of CRD-BP expression with these tumor-promoting molecules. Although tumor CRD-BP expression was not significantly correlated with tumor invasion, vessel involvement or tumor stage, CRC patients with CRD-BP expression showed trends for worse 5-year RFS and OS rates compared to those with no expression. Consequently, these results suggest that CRD-BP expression favors the progression of CRC via the stabilization of $\beta$-TrCP1 and c-myc.

In the present study, tumor CRD-BP mRNA expression was not significantly correlated with its protein expression level (Table 3), while tumor CRD-BP mRNA expression was significantly correlated with nuclear accumulation of $\beta$-catenin. The former finding contrasts with our previous biochemical study which showed significant associations in colon cancer cells and in a small subset of CRC cases (7). It was reported that CRD-BP is a target of let-7, a member of highly conserved group of micro (mi)RNAs that functions as a tumor suppressor (23), and that let-7 reduces CRD-BP expression (24, 25). A previous study reported that let-7 expression was decreased in colon cancer cells (26). Therefore, this miRNA may be partly involved in the observed dissociation in expression between CRD-BP mRNA and its protein in our present study. Compared with a previous immunohistochemical study showing cytoplasmic expression of CRD-BP in CRC tumor and normal colon crypt cells (13), the striking finding of our study is the prominent nuclear expression of CRD-BP in tumor cells in the majority of cases. This finding suggests that, in addition to its cytoplasmic fraction, nuclear fraction of CRD-BP may participate in mRNA stabilization, and thus result in higher levels of $\beta$-TrCP1, c-myc and IGF-II mRNA in the tumors than their corresponding normal mucosa tissues. We previously reported that CRD-BP stabilizes $\beta$-TrCP1 and c-myc mRNA, thereby inducing the expression of these proteins in colon cancer cells (7). It has also been reported by others that tumor cells with CRD-BP expression show increased expression of c-myc mRNA and protein (27). Hence, the present finding of the association of CRD-BP expression with increased $\beta$-TrCP1 and c-myc mRNA levels in clinical CRC is consistent with the previous studies (7, 25, 28).

A previous study in CRC showed that CRD-BP mRNA expression correlated with tumor size, presence of distant metastasis, tumor recurrence and risk of tumor-related deaths (13). Another study in ovarian cancer reported an association between tumor CRD-BP expression and poor prognosis (14). In a mouse model of CRC xenografts, IMP-1 (a homologue of CRD-BP) was shown to promote tumor growth, dissemination and a tumor-initiating cell phenotype (28). A trend towards poor prognosis for patients with tumor CRD-BP expression was observed in the present study of 42 CRC patients, however this did not reach statistical significance because of the small cohort scale and limited follow up time. Interestingly, we found significantly frequent expression of CRD-BP in younger (< 65 years) patients. Biological mechanism(s) for this preference is a matter of future analysis by focusing such as age-related epigenetic regulation of CRD-BP expression.
Several previous studies have shown that expression of CRD-BP (also described as ZBP-1, IGFII-BP1, IMP-1) in breast cancer (29), brain tumors, non-small cell lung cancer (30), choriocarcinoma (31), hepatocellular carcinoma (32) and malignant melanoma (15) acts functionally as an oncoprotein. It was also reported that CRD-BP was responsible for clonogenic propagation of breast cancer cells based on the increased expression of full-length CRD-BP, or of the N-terminal-truncated form lacking two RRM domains (12). In mammary-specific whey acidic protein (WAP) and CRD-BP transgenic mice that are prone to breast carcinogenesis, H19 RNA and IGF-II mRNA were upregulated in non-neoplastic breast tissue, suggesting that CRD-BP acts as a proto-oncogene (33). Moreover, it has been demonstrated that CRD-BP knock-down in primary and metastatic melanoma enhances the efficacy of various anti-cancer agents (dacarbazine, temozolomide, vinblastine and etoposide), suggesting that CRD-BP could be a potential therapeutic target and predictive biomarker for chemotherapeutic effect (34).

In a previous study using mouse models of CRC, IMP-1 overexpression increased the CRC xenograft volume and its dissemination into blood, while intestine-specific knock-down of IMP-1 in Apc Min/+ mice reduced the number of intestinal tumors (28). These results indicate that CRD-BP participates in the progression of CRC. Preliminary findings from another study suggest that activation of non-canonical NF-κB1 p105 signaling in CRC may be due to β-catenin-mediated induction of CRD-BP and β-TrCP1 (35).

Conclusions

As illustrated in Figure 6, the present findings together with our earlier results (7, 16, 22) indicate that CRD-
CRD-BP influences its target genes expression and clinical characteristics in CRC.

BP integrates the β-catenin-, NF-κB- and c-myc-mediated oncogenic pathways by stabilizing the mRNA for β-TrCP1, IxBα and c-myc in response to Wnt/β-catenin signaling, thereby promoting the progression of CRC. CRD-BP is therefore emerging as a critical factor in the understanding of CRC pathology, as well as a potential molecular target for the development of new treatment strategies against cancer.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (Y. T.) upon reasonable request.

Authors’ Contributions

YT, TM and TK formulated the design and concept of the study and drafted the manuscript. YT carried out the analysis and comparison of the expression of the molecules of interest and the data of CRC patients. TD and YU assisted and supported to carry out the analyses. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study has been performed in accordance with the Declaration of Helsinki. Tissues used in this study are from the patients diagnosed between 1998 and 2004 a written informed consent is available from all patients. The study protocol was reviewed and approved by the Kanazawa University Human Genome and Gene Analysis Research Ethics Committee (the reference No. 181). All samples were anonymized before analysis was performed to guarantee the protection of privacy.

Patient Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

References