Inhibition of Y Box Binding Protein 1 Suppresses Cell Growth and Motility in Colorectal Cancer

Aeurnuri Kim, Sehwan Shim, Young-heon Kim, Min-Jung Kim, Sunhoo Park, and Jae Kyung Myung

ABSTRACT

Although chemo- or radiotherapy is usually performed in patients with colorectal cancer, the response is highly variable in locally rectal cancer. Therefore, additional studies are needed on predictable markers and the molecular mechanisms of chemo- and radiotherapy. Y box binding protein 1 (YB1) is an oncoprotein that is aberrantly expressed in many cancers, including colorectal cancer. However, to date there are no targeting agents or strategies to inhibit YB1 expression. Here, we investigate the oncogenic function of YB1 in colorectal cancer and methods to control its expression. We observed that YB1 expression level is correlated with colorectal cancer survival rate. Moreover, YB1 overexpression was associated with colorectal cancer lymph node metastasis and invasion. We also found that radiation exposure increased YB1 expression, which led to radioresistant colorectal cancer, mediated through the activation of cancer stem cell marker CD44 and PI3K/AKT/mTOR signaling. This study revealed, by both in vitro and in vivo assays, that depletion of YB1 could reduce cell proliferation and motility in colorectal cancer. We further demonstrated that the PI3K/mTOR inhibitor BEZ235 suppressed YB1 expression and enhanced the cytotoxicity of radiation. In addition, combined treatment with BEZ235 and radiation showed a significant antitumor response in an in vivo mouse xenograft model. Taken together, our results provide evidence that the activation of YB1 is a major factor in radioresistance and suggest that targeting YB1-mediated signaling is a promising therapeutic strategy for colorectal cancer.

Introduction

Colorectal cancer is one of the most common malignancies worldwide (1). Surgery is the standard therapy for patients with colorectal cancer, and 20% of the patients show relapse (2). Thus, preoperative chemo- and radiotherapy are used commonly for colorectal cancer treatment to reduce recurrence (3). However, previous clinical studies have shown that chemo- and radiotherapy do not improve overall survival rate in locally rectal cancer; moreover, radiotherapy has been reported to promote distant metastases of rectal cancer (4, 5). Therefore, a deeper understanding of the molecular mechanics of radiotherapy resistance is needed for the development of potential therapeutic strategies to improve the survival of patients with colorectal cancer.

The Y box binding protein 1 (YB1) is a member of the cold-shock protein superfamily that contains DNA/RNA-binding proteins (6). YB1 is primarily localized in the cytoplasm, but various stimuli, such as DNA-damaging agents or irradiation (IR), can enhance the transcription factor activity of YB1 in the nucleus (7). For the nuclear accumulation and induction of transcriptional activity, YB1 must be phosphorylated at Ser102 (8). Phosphorylated and nuclear YB1 levels are a predictive factor for cellular response to DNA damage stressors, including IR (9). Nuclear YB1 can promote drug resistance and cancer progression through downstream targets including E2F, p53, PI3K/AKT/mTOR, and the RAS/RAF/MAPK pathway (10). In addition, YB1 is known to be associated with tumor cell invasion and migration through the regulation of epithelial–mesenchymal transition (EMT) molecules (11). Recent studies determined that YB1 could regulate cancer stem cell (CSC) markers, leading to tumorigenesis and metastasis during recurrence following chemo- and radiotherapy in breast cancer (12). Moreover, CD44 is well known as a cancer-initiating marker of colorectal cancer (13). Furthermore, studies report that high expression of YB1 is correlated with local recurrence and suggest this as an independent prognostic factor for colorectal cancer (14, 15). Based on these results, YB1 is an important marker of tumor cells that is highly expressed in cancers with poor prognosis and plays the role of an oncoprotein. YB1 has been suggested as a potential target for the therapeutic treatment of colorectal cancer (14–16); however, to date there are no therapeutic strategies that target YB1 and its downstream signaling pathway.

BEZ235 (Dactolisib) is a small-molecule dual inhibitor that specifically inhibits PI3K/mTOR (17). It is currently in phase I/II clinical trials and has shown potential as a target agent in preclinical mouse models of glioblastoma (18). In addition, many studies report that inhibition of PI3K/mTOR by BEZ235 overcomes resistance to conventional therapy in various cancer cells (19–21). Our previous study showed that BEZ235 enhanced the cytotoxicity of chemoaogents in colorectal cancers with KRAS and PIK3CA mutations in vitro and in vivo (22). However, the ability of BEZ235 to regulate YB1 and its associated signaling pathway remains unknown.

In this study, we determined that IR-induced YB1 promoted colorectal cancer tumorigenesis through the regulation of cell proliferation, motility, and invasiveness. Moreover, the downregulation of YB1 following BEZ235 treatment suppressed tumor growth and lymph node metastasis in vivo. Our findings suggest that targeting YB1 is a promising therapeutic approach in colorectal cancer, particularly in patients failing with radiotherapy.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Mol Cancer Ther 2020;19:479–89

doi: 10.1158/1535-7163.MCT-19-0265

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Materials and Methods

**Patient tissue microarrays and IHC**

Human tissue microarray slides containing 76 pairs of colorectal cancer tumor tissues and corresponding non-tumor tissues were purchased from ISU ABXIS. All slides were prepared with paraffin-embedded human tissues. Compared with the corresponding non-tumor tissues, 80 pairs of tumor tissues and its adjacent non-tumor colon tissues of colorectal cancer were obtained from the Korea Institute of Radiological and Medical Sciences, Seoul, Korea.

For the analysis of IHC scoring, the slide was stained with YB1 antibody (Cell Signaling Technology) and quantified based on the extent of staining. Tissue samples were prepared and stained as described previously (22). IHC score was determined independently by two pathologists. For analysis of IHC scoring, we used the formula: percentage of positive cells 3 staining intensity. Intensity was scored within a range of 0 to 3, representing negative, weak, moderate, and strong positivity, respectively. IHC of YB1 was calculated the percentage of positive-stained cells [0%–25% (1); 26%–50% (2); 51%–75% (3); 76%–100% (4)]. YB1 low was defined as the score of tumor tissues with 0% to 50%, and YB1 high as 51% to 100%. Images of immunostained slides were captured using a light microscope (Olympus).

**Cell culture, IR, and drug treatment**

The human colon cancer cell lines RKO, LoVo, SW480, SW620, DLD1, HCT116, and T84 were purchased from the American Type Culture Collection. These cell lines were cultured in RPMI 1640 media (Lonza) containing 10% FBS (Lonza) and maintained at 37°C with 5% CO2. The human rectal cancer cell lines SW837 and SW4163 were obtained from the ATCC and cultured with L15 media containing 10% FBS at 37°C with atmosphere. Cells were exposed to 137Cs γ-rays from a Gamma Cell-3000 irradiator (MDS Nordion International). BEZ235 was purchased by Selleckchem.

**Cell proliferation assay**

To measure cell growth and survival following treatment with BEZ235 or radiation, irradiated cells were plated in 60-mm dishes (RKO and SW620, 4 × 104 and DLD1 and SW837, 3 × 104) cultured for 3 days in media containing the indicated concentrations of BEZ235. Triplicate cultures of each cell type were incubated with fresh drug-free media for an additional 3 weeks to allow clonogenic growth. RKO cells were stained with 1% methyl blue in methanol, and colonies containing >50 cells were counted under a microscope (Leica). The survival fraction was calculated based on the survival of untreated and BEZ235-treated cells. Experiments were performed a minimum of 3 times.

**RNAi knockdown and plasmid transfection**

Lentiviral short hairpin RNAs (shRNA) targeting YB1 and controls were purchased from Sigma-Alrich. Transfection was performed using Oligofectamine (Life Technologies) following the manufacturer’s protocol. Lentivirus was generated by 293T cells with the shRNA vector using Lipofectamine 2000 and Plus reagents (Life Technologies). Viral particles were added to DLD1 and SW837 cells and then incubated for 2 days. The shRNA clones were selected with puromycin (2 μg/mL). To overexpress YB1, the plasmid pDEST-Myc-YBX1 was obtained from Addgene (plasmid 19878). SW620 cells were transiently transfected with the plasmid using Lipofectamine 2000. Cells overexpressing YB1 were selected with ampicillin.

**Migration and invasion assay**

Cells were seeded in 24-well chambers with an 8-μm pore insert either coated with Matrigel (BD Biosciences) for the invasion assay or uncoated for the migration assay. Following a 24-hour incubation, cells were fixed with 4% paraformaldehyde for 10 minutes. The noninvasive cells were removed, and the invasive cells were stained with crystal violet and imaged under a bright-field microscope (Olympus).

**Wound-healing assay**

To assess the wound repair, cells were seeded into 6-well plates, and the cell monolayer was scratched with a 200 μL pipette tip. Cells were washed and incubated with growth media for 12 hours under the indicated transfection and IR conditions. Images were taken under a bright-field microscope (Leica).

**Flow cytometric analysis**

Cells were treated with IR alone, BEZ235 alone, or a combination of both for 48 hours. To detect CD44 expression, cells were incubated with anti-CD44 conjugated to APC (BioLegend) for 20 minutes. To measure apoptotic cell death, cells were stained with Annexin V and propidium iodide (PI; BD Biosciences). Flow cytometric data were acquired on a FACSCanto (Becton & Dickinson) and analyzed using FlowJo software (TreeStar).

**Western blot analysis and nuclear extraction**

Western blot analysis was performed as described previously (22) using primary antibodies against the following proteins: p-YB1 (Ser102), p-AKT (Ser473), YB1, E-cadherin, CD44, vimentin, caspase-3, and cleaved PARP (Cell Signaling Technology); Ref1, AKT, mTOR, β-actin (Santa Cruz Biotechnology); p-mTOR and N-cadherin (Abcam). To detect nuclear YB1, nuclear protein was isolated using NE-PER. Nuclear and cytoplasmic extraction reagents and membrane protein were extracted by the Mem-PERTM Plus Kit in accordance with the manufacturer’s instructions (Thermo Scientific). Specific proteins were detected by Western blot.

**Immunofluorescence staining**

Samples were prepared and stained as described previously (22). To detect nuclear YB1 expression changes following treatment with IR, SW837 cells were exposed to IR (5 Gy). After the 24-hour IR, cells were fixed and stained with primary antibodies specific for YB1 (green), PI (red) was used for nuclear staining. To determine the colocalization of YB1 and CD44, DLD1 cells were treated with BEZ235 and IR for 24 hours. Expression was detected with anti-YB1 (green) and anti-CD44 (red; Cell Signaling Technology). Human intestinal organoids were treated with IR alone, BEZ235 alone, or a combination of both for 48 hours. To assess cell viability in organoids from patients with colorectal cancer, calcein (20 nmol/L; Sigma-Aldrich) and PI (0.2 μg/mL; Sigma-Aldrich) staining were used for 5 minutes at room temperature. Images of immunostained slides were captured using a confocal laser scanning microscope (Leica).

**In vivo tumorigenesis**

Six-week-old female BALB/c nude mice were obtained and maintained for 7 days. To examine intrahepatic tumorigenesis, SW837 (2 × 106) and SW837-shYB1 (2 × 106) cells were directly inoculated into the spleen of the mice. After 4 weeks, mice were sacrificed, and hepatic nodules >1 mm in diameter were counted. To investigate the anti-tumor effects of IR and BEZ235, SW837 cells (1 × 107) were s.c. injected into the flank of each mouse. One week after implantation, mice were randomly divided into four groups. BEZ235 was dissolved in...
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Primary human tissue collection and organoid culture

Tissues of patients with human colorectal cancer were obtained from the Korea Institute of Radiological and Medical Sciences. Colorectal cancer tissues were randomly collected from 10 patients diagnosed with colorectal cancer between 2017 and 2018. Experiments were approved by the ethics committee of IRB (Sub IRB NO: K-1702-002-059). Organoid culturing was performed according to the methods described by Sato and colleagues (23). Briefly, dissected biopsies were suspended in Matrigel (growth factor reduced; BD Biosciences) and dispensed into two 24-well culture plates. For Matrigel mixed samples, 30 μL was added per well. Advanced DMEM/F12 was supplemented with penicillin/streptomycin, 10 mmol/L HEPES, 2 mmol/L GlutaMAX, 1 × 827 (Life Technologies), and 1 mmol/L N-acetylcysteine (Sigma). Fifty nanograms per milliliter of human recombinant EGF (Life Technologies), 100 ng/mL human recombinant Noggin (Peprotech), 100 ng/mL human recombinant R-spondin-1 (Peprotech), 500 nmol/L A83-01 (Tocris), and 10 μmol/L SB202190 (Sigma) were added to the samples. Fresh media were added every 3 days. Outgrowing organoids were passaged every 10 to 14 days after mechanical and enzymatic disruption. The gels were overlaid with culture media supplemented with BEZ235. Following 72-hour treatment with BEZ235, organoids were exposed to IR (5 Gy) and then incubation for 48 hours.

Statistical analysis

Quantitative data are expressed as the mean ± SD. Mean values were compared using one-way ANOVA and Student t test. A P value < 0.05 was considered statistically significant. All experiments were repeated 3 times.

Results

YB1 overexpression was associated with a poor prognosis in patients with colorectal cancer

The protein level of YB1 was detected by IHC from 80 pairs of tumor and nontumor specimens of colorectal cancer. The nontumor tissues showed no expression nuclear YB1 expression, whereas high nuclear expression was observed in tumor samples (Fig. 1A). To evaluate the clinical significance of YB1, YB1 expression level was assessed by IHC using a commercially available tissue microarray for evaluation of 76 tumor tissue and nontumor samples from patients with colorectal cancer. Informative data were obtained from 156 pairs of tumor tissue and nontumor specimens of colorectal cancer. The nontumor tissues showed no expression nuclear YB1 expression, whereas high nuclear expression was observed in tumor samples (Fig. 1B). Next, radiography analysis revealed that YB1 expression was statistically significant association between tumor staging and high YB1 as well as a trend lymph node metastasis and invasion. Overall survival analysis revealed that YB1 expression was associated with worse outcome of patients with colorectal cancer (Kaplan–Meier method, P < 0.05, Fig. 1C). The YB1 expression level increased with colorectal tumor stage, implying that YB1 level affects the prognosis of patients with colorectal cancer. Together, these results indicate that upregulated YB1 is associated with high-grade tumors and suggest YB1 may be a prognostic marker for patients with colorectal cancer.

IR accelerated YB1 activation in colorectal cancer cells

We first evaluated phosphorylated YB1 (p-YB1) and total basal YB1 levels in various colorectal cancer cell lines. We found that both p-YB1 and total basal YB1 levels were increased in four of the colorectal cancer cell lines, including DLD1, T84, SW837, and SW1463 compared with the other colorectal cancer cell lines (Fig. 2A). In addition, nuclear YB1 was detected in RKO, SW620, DLD1, and SW837 colorectal cancer cell lines, and these levels were elevated in DLD1 and SW837 cells when compared with RKO and SW620 cells (Fig. 2B). Next, to determine whether YB1 levels affected IR response, we analyzed colony formation after colorectal cancer cell exposure to IR. As shown in Fig. 2C, DLD1 and SW837 cells were resistant to IR and exhibited elevated p-YB1 and nuclear YB1 when compared with RKO and SW620 cells. In contrast, p-YB1 and nuclear YB1 levels were decreased in radiosensitive RKO and SW620 cells (Fig. 2D and E). Immunofluorescence staining also revealed that IR exposure increased nuclear YB1 levels in SW837 cells (Fig. 2F). Taken together, these results indicate that elevated YB1 could be involved in colorectal cancer radioresistance.

YB1 depletion suppressed tumor cell growth in vitro and in vivo

Activated YB1 has the ability to enhance tumorigenesis and cell proliferation (24). To further explore the influence of YB1 expression on tumor cell growth, we depleted YB1 using specific shRNA-expressing lentiviruses in highly radioresistant cells. Knockdown of YB1 significantly inhibited the colony formation by 2-fold compared with shCont cells (Supplementary Data S1A). A xenograft formation assay was also performed to investigate whether YB1 depletion could suppress tumorigenesis in vivo. Tumor volume was substantially increased in mice with shCont DLD1 cells compared with shYB1 cells. Moreover, the IHC results showed lower YB1 and Ki67 expression in the tumors from mice inoculated with shYB1 when compared with those inoculated with shCont (Supplementary Data S1B). These results demonstrated that YB1 is associated with tumor growth in colorectal cancer in vitro and in vivo.

YB1 regulates the CSC markers CD44 and CD49f in breast cancer, leading to drug resistance (12). To determine whether YB1 directly regulates CD44 and CD49f expression in colorectal cancer cells, we treated DLD1, SW837, and their YB1-depleted counterparts with IR. As shown in Fig. 3A, p-YB1, YB1, and CD44 were reduced in shYB1 cells compared with shCont cells. In addition, IR increased p-YB1 and CD44 levels in both shCont cells, but not in YB1-depleted cells. Similar to this result, flow cytometry analysis showed that CD44 levels were greatly elevated by IR in shCont cells, while remaining constant in

1-methyl-2-pyrrolidone (NMP) and PEG300 (Sigma-Aldrich) to final concentrations of 10% NMP and 90% PEG300, respectively. Mice were orally administered 50 mg/kg BEZ235 (once daily for 21 days) or vehicle and exposed with 2 Gy IR (4 times for 2 weeks; given 2 hours after BEZ235). Mice were irradiated with an X-ray device (X-RAD 320) at a dose rate of 2 Gy/min. To determine metastasis ability following treatment with IR and BEZ235, SW837 (1 × 106) cells were injected into the footpad of mice. Three days after cell injection, mice were treated with IR alone, BEZ235 alone, or a combined treatment of IR and BEZ235. These mice were then exposed to IR two times (2 Gy/day) for a week and treated with 70 mg/kg BEZ235 once daily for 21 days by oral gavage. After 6 weeks, mice were sacrificed, and popliteal lymph nodes were isolated. Tumor and lymph nodes’ sizes in mice were measured with a caliper and were calculated using the formula: (length × width2) × 0.5. All animal studies were performed under protocols approved by the Korea Institute of Radiological and Medical Science.
shYB1 cells (Fig. 3B). However, CD49f levels were not significantly increased by IR, and it was not downregulated in YB1-depleted cells (Fig. 3A). Consistent with these results, YB1 depletion suppressed radioresistance, leading to an inhibition of colony formation in shYB1 cells (Fig. 3C). To confirm that YB1 is a major mediator of radiation resistance, we transfected radiosensitive SW620 cells with pDEST-Myc-YB1 to overexpress YB1. We observed that IR decreased p-YB1 and CD44 expression levels in pDEST-Myc vector cells, but it did not in YB1-overexpressing (pDEST-Myc-YB1) cells. As expected, IR treatment resulted in 33% apoptosis in control vector cells, whereas apoptosis was reduced to 16% in pDEST-Myc-YB1 cells (Fig. 3D). These results indicate that activated YB1 affects resistance to radiation through CD44 expression, implying that inhibition of YB1 enhances sensitivity to IR in colorectal cancer.

YB1 regulated migration and invasion of colorectal cancer cells in vitro and in vivo

Activated YB1 is associated with tumor progression through increased cell migration and invasion (11). To determine whether silencing YB1 blocks metastasis in vivo, we used a spleen-to-liver metastasis model. We inoculated shCont- and shYB1-treated DLD1 cells into mouse spleens to test metastasis capacity. As shown in Fig. 4A, shCont cell–bearing mice contained an average of 16 metastatic liver nodules, whereas shYB1-bearing mice contained an average of less than seven. Moreover, hematoxylin and eosin (H&E) staining showed that YB1-depleted cells significantly impeded tumor dissemination to the liver when compared with shCont cells. Thus, regulation of YB1 activity plays a critical role in colorectal cancer metastasis.

We next examined whether the activation of YB1 by IR could affect colorectal cancer cell motility. YB1-depleted cells were exposed to IR, and infiltration rates were analyzed utilizing transwell migration and invasion assays. YB1-depleted cells showed significantly lower (>50% reduction) migration and invasion rates in transwells when compared with control cells. As expected, IR exposure enhanced the antimetastatic ability of YB1-depleted cells, leading to a marked approximate 50% reduction in cell motility when compared with shCont cells (Fig. 4B). The wound-healing assay indicated that YB1 knockdown cells obtained slower closure when compared with control cells. Moreover, IR-exposed shCont cells almost recovered the wound, whereas IR-exposed shYB1 cells had significant unhealed wound area (Fig. 4C). Consistent with these findings, shYB1 cells showed increases in the epithelial marker E-cadherin and decreases in mesenchymal markers, including vimentin, N-cadherin, and fibronectin, when compared with shCont cells. In addition, IR did not elevate mesenchymal markers in YB1-depleted cells (Fig. 4D). Taken together with the above experimental evidence, downregulated YB1 blocked

Table 1. The correlation between YB1 expression level and clinicopathologic features of patients with colorectal cancer.

<table>
<thead>
<tr>
<th>Clinicopathology characteristics</th>
<th>Staining intensity Low, n (%)</th>
<th>Staining intensity High, n (%)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
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<tr>
<td>&lt;60</td>
<td>53 (33.97)</td>
<td>39 (25)</td>
<td>0.51</td>
</tr>
<tr>
<td>≥60</td>
<td>33 (21.15)</td>
<td>31 (19.87)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>48 (30.76)</td>
<td>37 (23.71)</td>
<td>0.33</td>
</tr>
<tr>
<td>Female</td>
<td>46 (29.48)</td>
<td>25 (16.02)</td>
<td></td>
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<tr>
<td>Staging</td>
<td></td>
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<td></td>
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<tr>
<td>0 and I</td>
<td>43 (27.56)</td>
<td>22 (14.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>II, III, and IV</td>
<td>31 (19.87)</td>
<td>60 (38.46)</td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>–</td>
<td>67 (42.94)</td>
<td>24 (15.38)</td>
<td>0.02</td>
</tr>
<tr>
<td>+</td>
<td>23 (14.74)</td>
<td>42 (26.92)</td>
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<tr>
<td>Tumor invasion</td>
<td></td>
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<tr>
<td>0–1</td>
<td>51 (32.69)</td>
<td>33 (21.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>24 (15.38)</td>
<td>48 (30.76)</td>
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IR-induced migration through a mesenchymal change in colorectal cancer cells.

**YB1 interacted with PI3K/AKT signaling in colorectal cancer**

Previous studies have shown that activated YB1 promotes cancer cell proliferation through several signaling pathways, including the PI3K/AKT/mTOR and MAPK/ERK pathways (25–27). We also found that IR increased phosphorylated AKT/mTOR and ERK levels in radiosensitive colorectal cancer cells compared with radiosensitive cells (Supplementary Data S2). We next determined whether activation of YB1 and its downstream signaling could induce radioresistance in colorectal cancer cells. To test this, we treated radioresistant DLD1 and SW837 cells with specific inhibitors, including PD98059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), and LY294002 (PI3K inhibitor). The PI3K inhibitor greatly reduced cell viability when compared with MEK/MAPK inhibitors in both cell lines. In addition, the inhibition of PI3K resulted in the downregulation of p-YB1 expression by Western blot 12 hours after IR (5 Gy). Ref1 was used as a loading control. These results indicate that YB1 is more closely associated with PI3K than the MEK/MAPK pathway in colorectal cancer cells. To determine whether YB1 interacts with the PI3K/AKT pathway, we

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**Figure 2.**

IR exposure affected phosphorylated and nuclear YB1 levels in colorectal cancer cells. A, Whole-cell lysates from eight colorectal cancer cell lines were analyzed for p-YB1 and YB1 levels using Western blotting. β-Actin was used as a loading control. B, Expression of YB1 was detected in the nuclear fraction of four colorectal cancer cell lines (RKO, SW620, DLD1, and SW837) by Western blot. Ref1 was used as a loading control. C, Colorectal cancer cells were exposed to the indicated doses of IR and then incubated for 14 days. Colony-forming assays of cells were performed, and colony number was calculated from three replicate plates of three independent experiments (bars indicate SD). Representative images from 14 days after plating are shown. D, Whole-cell lysates were used to assess p-YB1 and YB1 levels by Western blot following the IR (5 Gy) exposure of colorectal cancer cells at 6 and 12 hours. β-Actin was used as a loading control. E, Cells were evaluated for nuclear YB1 expression by Western blot 12 hours after IR (5 Gy). Ref1 was used as a loading control. F, Immunofluorescence staining for YB1 in SW837 cells 24 hours after IR (5 Gy) exposure. PI was used for nuclear staining.
performed knockdown of YB1 or AKT using siRNA in DLD1 and SW837 cells, followed by exposure to IR. The YB1 depletion significantly reduced the protein levels of p-YB1, YB1, and CD44. β-Actin was used as a loading control. Knockdown of AKT by siRNA showed downregulation of p-YB1, indicating an interaction between the PI3K/AKT pathway and YB1 (Supplementary Data S4B).

Downregulation of YB1 by BEZ235 enhanced radiosensitivity of colorectal cancer cells

To inhibit YB1 activation, we treated radioresistant DLD1 and SW837 cells with the dual PI3K/AKT/mTOR inhibitor BEZ235. As shown in Fig. 5A, IR alone increased p-YB1, p-AKT, p-mTOR, and CD44 expression levels, whereas combined treatment with IR and BEZ235 significantly decreased in DRD1 and SW837 cells. In particular, flow cytometry analysis revealed that IR-induced CD44 expression was reduced by combined treatment. Immunofluorescence staining also exhibited that both p-YB1 and CD44 were increased by IR, but the combined treatment with BEZ235 significantly downregulated these proteins in DLD1 cells. Next, we investigated whether the combined treatment with IR and BEZ235 affected the capacity of radioresistant cells to form colonies. Exposure to radiation resulted in a slight suppression of colony numbers, whereas combined treatment with IR and BEZ235...
inhibited colony formation in DLD1 and SW837 cells (Fig. 5B). Similarly, BEZ235 treatment enhanced the cytotoxic effects of IR, which resulted in increased apoptosis and induction of cleaved caspase-3 and PARP (Fig. 5C). These data indicate that BEZ235 could reduce YB1 and CD44 activation, which would enhance radiation-induced cytotoxicity in colorectal cancer cells. To further investigate whether treatment with BEZ235 affects motility, we detected EMT marker expression in radioresistant cells. As shown in Fig. 5D, BEZ235 alone and combined treatment showed upregulation of E-cadherin and repression of vimentin, N-cadherin, and fibronectin when compared with IR alone. In line with this, a cell migration assay revealed that combined treatment significantly decreased the number of migrated cells (Fig. 5E). This evidence showed that inhibition of YB1 by BEZ235 suppressed colorectal cancer cell motility.

Combined treatment with BEZ235 and IR promoted antitumor effects in vivo

The above results demonstrated that inhibition of YB1 by BEZ235 can overcome radioresistance in colorectal cancer cells. To explore whether combined treatment suppressed tumor growth in vivo, SW837-bearing xenograft mice were s.c. injected. To assess the antitumor effects, mice were treated with BEZ235 (50 mg/kg) or IR (2 Gy × 4 times). Like the in vitro data, combined treatment effectively suppressed tumor volume when compared with the vehicle control or single-treatment groups (Fig. 6A). Unlike SW837-bearing mice, tumor volume was sufficiently reduced by a single treatment in YB1–expressed SW620-bearing mice (Supplementary Data S5). IHC staining revealed that combined treatment decreased Ki67, YB1, and CD44 expression and increased levels of cleaved caspase-3 when compared with their single-treatment counterparts (Fig. 6B).
determine whether BEZ235 and IR can inhibit tumor metastasis in vivo, SW837 cells were inoculated into the footpads of mice that were then treated with IR and BEZ235 with respect to the previously established treatment groups. Six weeks after injection, the mice were sacrificed and inguinal lymph nodes were obtained. The lymph node volumes significantly decreased with the combined treatment when compared with the single-treatment counterparts. Moreover, to confirm infiltrated cancer cells in the lymph nodes, we stained samples with pan-cytokeratin, which indicates metastatic epithelial tumor cells. The lymph nodes from mice with the combined treatment showed smaller volumes (Supplementary Data S6) and fewer pan-cytokeratin–positive tumor cells than those in the untreated or single-treatment groups (Fig. 6C). To investigate whether combined treatment increased cytotoxicity in organoids derived from patients with colorectal cancer, we treated organoids with IR and BEZ235, alone and in combination, for 48 hours. Single treatment with IR and BEZ235 slightly reduced organoid formation, but their combined treatment greatly inhibited organoid formation. We also analyzed survival rates using calcine and PI staining. As expected, representative images showed that combined treatment enhanced cytotoxicity in organoids when compared with single-treatment groups (Fig. 6D). Collectively, treatment with BEZ235 downregulated YB1 and CD44 expression, which enhanced the cytotoxicity of IR in vitro and in vivo, suggesting that a combined treatment has therapeutic potential for radioresistant patients with colorectal cancer.

Discussion

In the current study, we demonstrate that elevated YB1 phosphorylation progresses colorectal cancer tumor growth and cell motility both in vitro and in vivo. We found that YB1 increases radioresistance in colorectal cancer cells through the upregulated expression of the CSC marker CD44. In addition, the dual AKT/mTOR inhibitor BEZ235 inhibited YB1 activation, leading to a suppression of tumor growth and metastasis in mice- and human-derived colorectal cancer organoids. Our data indicate that the overexpression of YB1 serves as a potential prognostic marker and suggests that treatment with BEZ235 is a potential therapeutic strategy to overcome IR resistance in patients with colorectal cancer.

Radiotherapy is commonly used in patients with cancer, including skin, head and neck, as well as breast cancer. It is also performed as a neoadjuvant or adjuvant therapy to reduce the risk of recurrence in almost all patients with colon cancer and localized rectal cancer (3). However, the treatment response is highly variable and often results in minimal regression or even tumor progression (4, 5). In addition, a previous clinical study showed that conventional chemo- and
radiotherapy could not improve overall survival in localized rectal cancer (4). Thus, to improve radiation response rates, specific targeted therapies are needed for patients with colorectal cancer with radioresistance.

Elevated YB1 expression is known to be correlated with cancer progression and poor prognosis in many cancers, including lung, prostate, breast, gastric, glioblastoma, and sarcoma (11, 24–31). A previous study by Jin and colleagues suggested that elevated YB1 was associated with local recurrence and may be an independent prognostic marker for patients with colorectal cancer (14). Zhang and colleagues also reported that YB1 showed higher overexpression in rectal cancer tissue than in rectal tubular adenoma, implying that YB1 levels are correlated with tumor stage (15). In addition, nuclear YB1 expression may be associated with poor clinical outcomes in patients with stage III colorectal cancer (16). Our results showed that YB1 was significantly elevated in tumor tissues when compared with normal
tissues. It was also more highly expressed in metastatic and invasive tumor tissues when compared with nonmetastatic tumor cells. Furthermore, YB1 expression was increased in high-grade tumors and affected the overall survival status of patients with colorectal cancer. Taken together, these results imply that upregulated YB1 may be a marker for poor prognosis in colorectal cancer.

Phosphorylation of YB1 at Ser102 is essential for its nuclear accumulation and transcriptional function (32). Previous studies have shown that phosphorylated YB1 is overexpressed in IR-resistant cells, leading to the repair of DNA double-strand breaks and survival in breast cancer cells (9). Our results showed that IR significantly increased both phosphorylated and nuclear YB1 levels in radioreistant cells when compared with radiosensitive colorectal cancer cells. These results suggest that YB1 expression could be regulated by IR, which may enhance the malignant potential of colorectal cancer cells and could be utilized as a prognostic marker after radiotherapy. Therefore, an anti-YB1 strategy may provide a rationale for the successful therapeutic benefit of combination therapy.

YB1 is a multifunctional cancer marker, which directly or indirectly regulates several downstream targets (6). A previous study by Dunn and colleagues showed that YB1 promotes cancer cell growth and drug resistance through an analysis of CSC markers CD44 and CD49f in breast cancer (12). Indeed, CD44 is widely considered a major CSC resistance marker in colorectal cancer cells (9). Our results showed that IR significantly increased both phosphorylated and nuclear YB1 levels in radioreistant cells when compared with radiosensitive colorectal cancer cells. These results suggest that YB1 expression could be regulated by IR, which may enhance the malignant potential of colorectal cancer cells and could be utilized as a prognostic marker after radiotherapy. Therefore, an anti-YB1 strategy may provide a rationale for the successful therapeutic benefit of combination therapy.

YB1 is an oncogene that promotes cancer cell proliferation and survival through an interaction with several pathways, including E2F, Rb, p53, PI3K/AKT/mTOR, and MEK/ERK (10, 27). Our data showed that PI3K inhibition significantly reduced cell viability and p-YB1 expression when compared with MEK/ERK inhibition in colorectal cancer cells. The activation of PI3K/AKT/mTOR signaling is known to be involved in the radioresistance of many cancer cells (34, 35). We showed that radiation exposure increased AKT/mTOR expression in colorectal cancer cells. Moreover, lymph node metastatic cancer–derived organoids from patients with colorectal cancer showed highly expressed YB1 and CD44, compared with nonmetastatic organoids (Supplementary Data S7). Therefore, radioresistance can be overcome through the downregulation of YB1; however, until now, there was no known target agent for YB1 suppression.

YB1 is an oncogene that promotes cancer cell proliferation and survival through an interaction with several pathways, including E2F, Rb, p53, PI3K/AKT/mTOR, and MEK/ERK (10, 27). Our data showed that PI3K inhibition significantly reduced cell viability and p-YB1 expression when compared with MEK/ERK inhibition in colorectal cancer cells. The activation of PI3K/AKT/mTOR signaling is known to be involved in the radioresistance of many cancer cells (34, 35). We showed that radiation exposure increased AKT/mTOR expression in radioresistant cancer cells when compared with radiosensitive cells. We also confirmed that the depletion of YB1 by siRNA suppressed the AKT/mTOR pathway in the IR-exposed colorectal cancer cells. In addition, knockdown of AKT can reduce YB1 activation in radioresistant colorectal cancer cells with or without IR. These results indicate that YB1 has a greater interaction with the PI3K/AKT/mTOR pathway when compared with the MEK/ERK pathway, leading to colorectal cancer cell growth and migration. Taken together, PI3K/mTOR inhibition could regulate YB1 and provide a rationale for further studies to enhance the therapeutic benefit of radiotherapy in a clinical setting.

BEZ235 is a small-molecule inhibitor that dually suppresses PI3K/mTOR. Previous studies have reported that BEZ235 exhibited anti-tumor activity in breast, glioma, and pancreatic cancer (36–38). Treatment with BEZ235 potently inhibits both ATM and DNA-PKcs, resulting in the radiosensitization of glioblastoma cell lines (18). The inhibition of hypoxia-inducible factor-1α by BEZ235 increased cancer cell apoptosis, suggesting that combined treatment including BEZ235 and radiation or chemotherapy could be beneficial for patients (39). Our results showed that BEZ235, treatment time and dose dependently, reduced the expression of AKT/mTOR and p-YB1 in colorectal cancer cells (Supplementary Data S8). In addition, combined treatment with BEZ235 and IR led to a significant suppression of YB1 expression, which reduced cell viability by caspase-mediated apoptosis and cell mobility by mesenchymal markers. Consistent with this, the combined treatment greatly increased the antitumor effect of either treatment alone, suppressing tumor size and lymph node metastasis in xenograft mouse model. Furthermore, we found that combined treatment with BEZ235 and IR significantly induced growth inhibition and death in organoids derived from patients with colorectal cancer. We previously reported that BEZ235 inhibits PI3K/mTOR signaling in colorectal cancer cells, leading to an enhancement of the cytotoxic effects of conventional chemotherapies (22). However, the reason why targeting PI3K/AKT/mTOR is effective in colorectal cancer has not been fully addressed.

In this study, we demonstrate that YB1 levels were significantly elevated in patients with high-stage colorectal cancer. Treatment with BEZ235 inhibited IR-induced YB1 expression and activation of the downstream AKT/mTOR pathway, which enhanced the cytotoxicity of IR in colorectal cancer both in vitro and in vivo. Taken together, anti-YB1 strategies may provide an opportunity to improve the outcome for patients with radioresistant colorectal cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Kim, S. Park, J.K. Myung
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Shim, Y.-h. Kim
Writing, review, and/or revision of the manuscript: A. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Shim, Y.-h. Kim

Acknowledgments
We thank Drs. Ki Moon Seong and You Yeon Choi for their insightful discussions. We thank Hye Won Kim and Sun Joo Lee for the technical assistance in the animal experiments.

This study was supported by grants from the Korea Institute of Radiological and Medical Sciences (KIRAMS), funded by Ministry of Science and ICT (MSIT), Republic of Korea (Nos. 50476-2019 and 50535-2019).

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Received March 12, 2019; revised August 14, 2019; accepted October 25, 2019; published first October 31, 2019.

References


Molecular Cancer Therapeutics

Inhibition of Y Box Binding Protein 1 Suppresses Cell Growth and Motility in Colorectal Cancer

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doi:10.1158/1535-7163.MCT-19-0265

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