RYBP contributes to improved prognosis in colorectal cancer via regulation of cell cycle, apoptosis and oxaliplatin sensitivity

TAKASHI MORINAKA[1], NOZOMU SAKAI[1], TSUKASA TAKAYASHIKI[1], SATOSHI KUBOKI[1], SHIGETSUGU TAKANO[1], GAKU OHIRA[2], HISAHIRO MATSUBARA[2] and MASAYUKI OHTSUKA[1]

Departments of [1]General Surgery and [2]Frontier Surgery, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

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Abstract. Ring1 and YY-1 binding protein (RYBP) is a member of the polycomb repressive complex 1 and serves as a transcriptional suppressor via epigenetic modification. RYBP has a tumour-suppressive role in solid tumours, but its function in colorectal cancer (CRC) remains unknown. The present study evaluated the expression of RYBP using immunohistochemistry in 140 cases of primary CRC and 11 patient-matched cases of liver metastases. Using CRC cell lines with different *TP53* gene status such as HCT116 ($TP53^{wt/wt}$), HCT116 ($TP53^{-/-}$), SW48 and DLD-1 cells, proliferation, cell cycle progression and apoptosis, as well as the effect of RYBP on oxaliplatin sensitivity, were assessed. Clinical data showed that low RYBP expression was significantly associated with risk of distant metastasis and recurrence, and patients with high RYBP expression demonstrated significantly better cancer-specific and disease-free survival. *In vitro* experiments revealed that RYBP suppressed cell proliferation by inducing cell cycle arrest and apoptosis in *TP53* wild-type cells. In addition, endogenous RYBP overexpression enhanced sensitivity to oxaliplatin. Therefore, RYBP may contribute to improved prognosis in CRC by regulating the cell cycle, apoptosis and oxaliplatin sensitivity via the p53-mediated pathway.

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related deaths (1). A total of ~35% of patients with CRC are initially diagnosed with metastasis and 20-50% of patients with non-metastatic CRC develop metastasis during the course of disease (2,3). Although efforts have been made to elucidate molecular pathways associated with CRC progression (4-6), metastasis of CRC is a major factor responsible for poor prognosis and its treatment remains challenging. Therefore, better understanding of the molecular mechanisms underlying CRC metastasis is required.

Ring1 and YY-1 binding protein (RYBP) was originally identified as a member of the polycomb repressive complex (7). RYBP epigenetically regulates gene expression and is involved in embryonic development, stem cell self-renewal, cell differentiation, and X chromosome inactivation (7-9). RYBP interacts with several transcription factors, including YY-1, and serves as a bridge factor that mediates the formation of transcription factor complexes, thereby regulating gene expression independent of polycomb group functions (10,11). RYBP mediates the interaction of YY-1 with the E2F family of genes, which encode transcription factors that serve an important role in G1/S phase transition (11). Our previous study demonstrated that YY-1 acts as a tumour suppressor and high YY-1 expression contributes to improved CRC prognosis (4). RYBP may also act as a tumour suppressor. Previous studies have demonstrated that RYBP plays tumour-suppressive roles in several types of cancer, such as hepatocellular carcinoma (12) and breast (13), lung (14) and oesophageal cancer (15). To the best of our knowledge, however, there have been no functional analyses or clinicopathological studies of RYBP with regards to CRC. Therefore, the present study aimed to evaluate the role and expression of RYBP in CRC, its association with clinical outcomes and underlying molecular mechanisms.

Materials and methods

Patients and specimens. Primary CRC tissues were obtained from 140 consecutive patients aged 27-91 years (49 females and 91 males who underwent surgical resection between January 2012 and December 2013 at Chiba University Hospital (Chiba, Japan). Corresponding liver metastasis tissue was obtained from 11 matched patients who underwent surgical resection at Chiba University Hospital. These patients ranged in age from 48 to 76 years, and consisted of 5 females and 6 males. Cases in which preoperative chemotherapy resulted in the total disappearance of tumour cells were excluded. Patients who underwent two-stage hepatectomy were excluded. The

Correspondence to: Dr Nozomu Sakai, Department of General Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
E-mail: sakain@chiba-u.jp

Abbreviations: CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival; IHC, immunohistochemistry; RYBP, ring1 and YY-1 binding protein

Key words: RYBP, tumour suppressor, cell cycle, apoptosis, chemoresistance

clinicopathological characteristics of patients are shown in Table SI. The Ethics Committees of the Department of General Surgery, Chiba University Hospital (Chiba, Japan) approved the study protocol (approval no. M10101) and written informed consent to participate was obtained from each patient before surgery. Union for International Cancer Control TNM classification of 8th edition (16) was used to assess the clinical outcome of the patients with CRC.

Immunohistochemistry (IHC). Paraffin-embedded tissue samples after 10% formalin fixed for 24 h at room temperature were cut to obtain 4-μm-thick slices and de-paraffinized with xylene and rehydrated with descending ethanol series. Antigen retrieval was performed by microwaving (500 W) slides in citric acid buffer (0.01 M; pH, 6.0) for 25 min at 100˚C. Subsequently, endogenous peroxidase activity was blocked with hydrogen peroxide (3% in methanol) for 15 min at room temperature. Non-specific proteins were blocked with 5% bovine serum albumin (BSA; cat. no. 01860-36; Nacalai Tesque, Inc.) for 10 min at room temperature. Slides were incubated overnight at 4˚C with the following primary antibodies: Anti-RYBP polyclonal (1:200; cat. no. HPA053357; Sigma-Aldrich; Merck KGaA) and anti-p53 Ab (1:100; cat. no. M7001; Dako; Agilent Technologies, Inc.). Secondary antibodies (undiluted; EnVision™ kits; cat. nos. K4001 and K4003; Dako; Agilent Technologies, Inc.) were applied for 30 min at room temperature, followed by staining with peroxidase DAB kit (cat. no. 25985-50; Nacalai Tesque, Inc.). Counterstaining was performed with haematoxylin before dehydration for 1 min at room temperature, penetration and mounting.

Using an inverted light microscope (cat. no. BX40; Olympus Corporation), the expression levels of RYBP and p53 were independently evaluated by two investigators and a pathologist, all of whom were blinded to clinical information. A 1% BSA/phosphate-buffered saline (PBS) solution without primary antibody served as a negative control. As a positive control, staining of normal liver tissue that originated from the tissue at least 1cm away from the tumour in a resected specimen from a patient with colorectal liver metastasis was confirmed because RYBP is expressed in the cytoplasm of normal hepatocytes (12). The evaluations were performed after establishing an inter-observer consensus using samples from preliminary experiments. In primary CRC, RYBP expression was evaluated by the percentage of positively stained nuclei in tumour cells relative to the total number of malignant cells in three positive high-power fields which were randomly selected (magnification, x400); low and high expression were defined as <20 and ≥20%, respectively. The expression of p53 was evaluated by considering the distribution pattern of positive cells and the intensity of nuclear staining and categorised as wild-type (wt; absent and weakly and sporadically positive staining) and mutant p53 (strongly mosaic and diffuse staining).

Human colon cancer cell lines and culture. The human colon cancer cell lines HCT116 (*TP53*$^{wt/wt}$) and HCT116 (*TP53*$^{-/-}$) were kindly provided by Dr Mamoru Takada (Chiba University, Chiba, Japan). SW48, DLD-1, HT29, COLO201 and SW620 were purchased from American Type Culture Collection. HCT116 cells were cultured in McCoy's 5A medium (cat. no. 16600082) with 10% foetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37˚C with 5% CO_2. SW48 and SW620 cells were cultured in Leibovitz's L-15 medium (cat. no. 11415064; Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS and incubated at 37˚C without CO_2. DLD-1 and COLO201 cells were cultured in RPMI 1640 medium (cat. no. 22400089; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and incubated at 37˚C with 5% CO_2.

Small interfering (si)RNA and plasmid transfection. HCT116 (*TP53*$^{wt/wt}$), HCT116 (*TP53*$^{-/-}$), SW48 and DLD-1 cells (2-5x10^5/well) were transfected with siRNA-1 and siRNA-2 [ON-TARGETplus Human RYBP (23429) siRNA, cat.no. J-015936-06 (target; 5'-GAAAGAUCCUCCUAGUGAA-3') and J-015936-07 (target; 5'-CGACAUGUCAGCAGUCAAU-3'); both GE Healthcare Dharmacon, Inc.] at a final concentration of 10 nmol/l or an equimolar concentration of control siRNA (AllStars Negative Control siRNA; sequence not available; cat. no. 1027280; Qiagen GmbH.) using Lipofectamine™ RNAiMAX transfection reagent (cat. no. 13778075; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h according to the manufacturer's recommendations. The expression (pcDNA3.1-RYBP) and empty vector (pcDNA3.1) were purchased from GenScript. Plasmid transfection was performed using 1 μg pcDNA and Lipofectamine® 3000 transfection reagent (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h incubation, according to the manufacturer's instructions. Changes in RYBP levels were monitored using western blotting 48 h after transfection. The cells were used for subsequent assays 24 h after transfection.

Western blot analysis. Proteins were extracted from cultured cells using RIPA buffer (cat. no. 16488-34; Nacalai Tesque, Inc.). Each protein sample was lysed in Laemmli Sample Buffer (cat. no. 1610737; Bio-Rad Laboratories, Inc.) containing 5% 2-mercaptoethanol and incubated at 97˚C for 10 min. After measuring the protein concentration of each sample using the Pierce™ BCA Protein Assay kit (cat. no. 23225; Thermo Scientific; Thermo Fisher Scientific, Inc.), 20 μg/lane protein was separated by electrophoresis on 10% XV PANTERA Gels (cat. no. NXV-224P; DRC) and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in 5% skimmed milk diluted in 0.1% Tris-buffered saline with Tween-20 or Blocking One-P (cat. no. 05999-84; Nacalai Tesque, Inc.) in the case of phosphorylated protein at room temperature (15-25˚C) for 60 min. The membranes were incubated at 4˚C overnight with primary antibodies against RYBP (1:1,000; cat. no. HPA053357; Sigma-Aldrich; Merck KGaA), p53 (1:1,000; cat. no. M7001; Dako; Agilent Technologies, Inc.), p21 (1:1,000; cat. no. 2947; Cell Signaling Technology, Inc.), cyclin D1 (1:1,000; cat. MA5-14512; Thermo Fisher Scientific, Inc.), Bax (1:1,000; cat. no. 5023; Cell Signaling Technology, Inc.), p-cyclin D1 (1:1,000; cat. no. 3300; Cell Signaling Technology, Inc.), YY-1 (1:1,000; cat. no. ab109228; Abcam) and β-actin (1:2,000; cat. no. 5125; Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with secondary antibodies at room temperature (15-25˚C) for 60 min as follows: Anti-mouse (1:2,000; cat. no. SC516102; Santa Cruz Biotechnology, Inc.) and anti-rabbit

HRP conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.). The membranes were incubated with an enhanced chemiluminescence detection reagent (Chemi-Lumi One Ultra; Nacalai Tesque, Inc.) and developed using a LAS-4000UV mini luminescent image analyser (FUJIFILM Wako Pure Chemical Corporation). Band intensities were quantified by densitometric analysis using the ImageJ software version 1.53 (National Institutes of Health) to calculate the relative protein levels normalised to β-actin.

Cell proliferation assay. HCT116 (*TP53*$^{wt/wt}$), HCT116 (*TP53*$^{-/-}$), SW48 and DLD-1 cells were seeded in 96-well microplates at a density of 3,000 cells/well. At 0, 24, 48, 72, or 96 h, 10 μl Cell Count Reagent SF (cat. no. 07553044; Nacalai Tesque, Inc.) was added to 100 μl culture medium. After 2 h incubation at 37˚C, the absorbance at 450 nm was measured using a microplate reader.

Cell cycle assay. HCT116 (*TP53*$^{wt/wt}$), HCT116 (*TP53*$^{-/-}$) cells were collected 48 h after transfection, with or without 24 h pre-treatment with 4 or 10 μM oxaliplatin (Nippon Kayaku Co., Ltd.) at 37˚C, washed with ice-cold PBS and fixed in 70% ethanol at -30˚C overnight. Prior to staining, cells were washed with PBS. Pellets of the cells were mixed with 500 μl FxCycle™ PI/RNAse Solution (cat. no. 10797; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 30 min at room temperature. Samples were analysed by fluorescence-activated cell sorting using FACS Canto II (BD Biosciences). All data were analysed using FlowJo v10.7.1 software (BD Biosciences).

Cell apoptosis assessment. Cell apoptosis was assessed using the FITC Annexin V/PI Apoptosis Detection kit (cat. no. 15342-54; Nacalai Tesque, Inc.), according to the manufacturer's instructions. Briefly, HCT116 (*TP53*$^{wt/wt}$), HCT116 (*TP53*$^{-/-}$) cells were collected, washed with Binding Buffer at 4˚C and resuspended in 100 μl Binding Buffer. A total of 1x10^5 cells were incubated with 5 μl Annexin V-FITC and PI at room temperature in the dark for 15 min. In total, 10,000-20,000 cells were analysed on a FACS Canto II (BD Biosciences) using FlowJo v10.7.1 (BD Biosciences). Oxaliplatin-treated cells were used as a positive control. The percentage of apoptotic cells was calculated based on the number of early apoptotic cells which were Annexin V-positive and PI negative.

Cell viability assessment. HCT116 (*TP53*$^{wt/wt}$), HCT116 (*TP53*$^{-/-}$) cells were seeded in 96-well microplates at a density of 5,000 cells/well and 100 μl of cell-free McCoy's 5A medium was prepared as a blank. After 24 h pre-incubation at 37˚C, oxaliplatin was added at concentrations of 0.001, 0.010, 0.1, 0.5, 1, 2, 4 and 10 μM and cells were incubated for 2 days at 37˚C. A total of 10 μl Cell Count Reagent SF (cat. no. 07553044; Nacalai Tesque, Inc.) was added to each well. After 2 h incubation, absorbance at 450 nm was measured using a microplate reader. The cell viability was calculated as follows: Cell viability (%)=(absorbance of treated sample-absorbance of cell-free sample)/(absorbance of control sample-absorbance of cell-free sample) x100.

The half-maximal inhibitory concentration (IC_{50}) was calculated with a non-linear regression fit to a sigmoidal dose-response curve and compared using the extra-sum-of-squares F test in Prism 9 (GraphPad Software, Inc.; Dotmatics). Experiments were performed six times independently.

Statistical analysis. Data are expressed as mean ± standard error of the mean or median ± standard deviation. The association between RYBP or p53 staining and patient characteristics were evaluated using χ^2 for categorical variables, unpaired Student's t for parametric continuous variables or Mann-Whitney U test for nonparametric continuous variables. Survival rates were calculated using Kaplan-Meier analysis and assessed using the log-rank test. Survival data were evaluated using univariate and multivariate Cox proportional hazards regression analysis. When analysing the association between RYBP expression in primary tumour and long-term outcomes, cancer-specific survival (CSS) and disease-free survival (DFS) were calculated from the date of primary tumour resection. When analysing the association between RYBP expression in liver metastases and long-term outcome, CSS and DFS were calculated from the date of initial hepatectomy. The *in vitro* experiments were performed at least three times independently and data were analysed using unpaired Student's t test or one-way or multivariate analysis of variance followed by Tukey's post hoc test. $P \leq 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using the JMP® Pro15 software (SAS Institute Inc.).

Results

RYBP expression in primary tumour is associated with better prognosis. To investigate RYBP expression and subcellular localisation in CRC, immunohistochemical staining of primary CRC and liver metastases was performed. In primary CRC, RYBP was predominantly expressed in the nuclei of both tumour and adjacent normal tissue (Fig. 1A). High RYBP expression was observed in 76 (54.3%) patients. The association between RYBP expression profiles and clinicopathological features is shown in Table I. The rate of distant metastases and recurrence after primary surgery were significantly higher in patients with low RYBP expression than in those with high RYBP expression (Table I). Kaplan-Meier analysis showed that patients with high RYBP expression had a significantly longer CSS and DFS after primary surgery than those with low RYBP expression (Fig. 1B). Univariate and multivariate analyses revealed that poor pathological differentiation, severe lymphatic invasion and low RYBP expression were independent prognostic factors associated with CSS (Table II).

Association between RYBP expression and better prognosis depends on p53 expression status. Wt p53 expression was observed in 70 patients (50%), whereas mutant p53 expression was observed in 70 patients (50.0%; Fig. 1C). The status of p53 expression was not associated with clinicopathological features or prognosis (Table III). In patients with wt p53, CSS and DFS were significantly longer in those with higher RYBP expression (Fig. 1D). By contrast, in patients with mutant p53 expression, there were no significant differences in CSS and DFS (Fig. 1D). These data indicated that RYBP contributed to better prognosis in a *TP53* status-dependent manner.

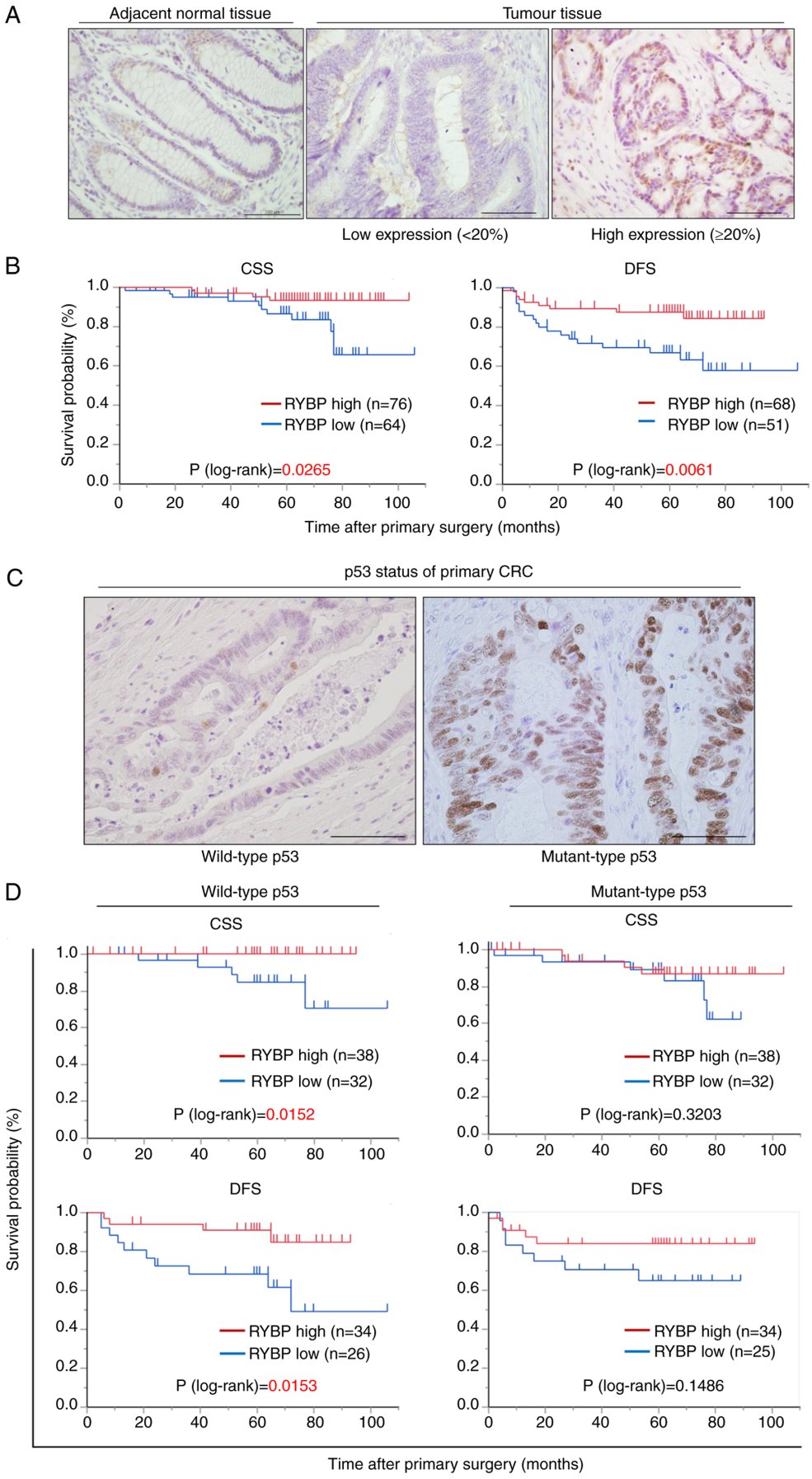


Figure 1. Expression of RYBP and p53 in primary CRC. (A) Expression of RYBP in primary CRC and adjacent normal tissue. (B) Kaplan-Meier analysis for CSS and DFS of patients with CRC based on RYBP expression in the nucleus. (C) Expression of p53 in primary CRC tissue. (D) Kaplan-Meier analysis for CSS and DFS of patients with CRC and wild-type and mutant p53 based on RYBP expression levels. Scale bar, 100 μm. RYBP, RYBP, ring1 and YY-1 binding protein; CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival.

Table I. Association between RYBP expression and clinicopathological features of patients with colorectal cancer.

Characteristic	RYBP expression		P-value
	High (n=76)	Low (n=64)	
Median age, years (range)	70 (29-91)	69 (27-91)	0.5608
Sex, female/male	25/51	24/40	0.5693
Primary lesion site, colon/rectum	42/34	40/24	0.3865
Primary lesion site, right colon/left colon/rectum	9/33/34	3/37/24	0.1385
CEA, <5/≥5 ng/ml	50/26	33/31	0.0878
CA19-9, <37/≥37 U/ml	60/16	54/10	0.4107
Postoperative chemotherapy, no/yes	53/23	42/22	0.6038
T stage[a], 1-3/4	60/16	45/19	0.1616
Lymph node metastasis, no/yes	51/25	33/31	0.0615
Ly, 0/1-3	34/42	27/37	0.7619
V, 0/1-3	33/43	26/38	0.7386
Degree of differentiation, pap or tub/por or muc	72/3	63/1	0.3916
KRAS, wild-type/mutant	6/13	10/16	0.6338
BRAF, wild-type/mutant	0/19	2/24	0.2162
Distant metastasis, no/yes	63/13	38/26	0.0019[b]
Recurrence after primary surgery, no/yes	63/5	38/13	0.0062[b]

[a]Union for International Cancer Control 8th edition. [b]P<0.01. RYBP, ring1 and YY-1 binding protein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; pap, papillary adenocarcinoma; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma; Ly, lymphatic invasion; V, Vascular invasion.

Table II. Univariate and multivariate analysis of cancer-specific survival in patients with colorectal cancer.

Variable	n	5-year survival, %	Univariate P-value	Multivariate	
				HR (95%CI)	P-value
Age, 65</≥65 years	43/97	86.7/92.2	0.307		
Sex, male/female	91/49	85.1/100.0	0.155		
CEA, <5/≥5 ng/ml	83/57	93.1/86.0	0.181		
CA19-9, 37</≥37 U/ml	114/26	93.6/74.5	0.050		
Site of primary tumour, left/right colon	128/12	90.5/88.9	0.867		
T stage[a], 1-3/4	105/35	91.0/87.5	0.337		
Degree of differentiation, tub or pap/por or muc	135/4	91.8/50.0	0.004[c]	7.46 (1.32-42.26)	0.023[b]
Ly, 0-1/2-3	121/19	95.6/60.8	<0.001[d]	5.17 (1.63-16.42)	0.005[c]
V, 0-1/2-3	96/44	94.7/81.1	0.027[b]	2.46 (0.82-7.41)	0.109
Lymph node metastasis, no/yes	84/56	96.6/81.7	<0.001[d]	2.98 (0.56-15.78)	0.199
RAS, wild-type/mutant	29/16	75.2/67.1	0.448		
Expression of RYBP, high/low	76/64	93.3/86.8	0.027[b]	3.77 (1.02-13.90)	0.046[b]

[a]Union for International Cancer Control 8th edition. [b]P <0.05, [c]P <0.01, [d]P<0.001. RYBP, ring1 and YY-1 binding protein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; pap, papillary adenocarcinoma; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma; HR, hazard ratio; CI, confidence interval; Ly, lymphatics invasion; V, Vascular invasion.

RYBP expression is downregulated in matched liver metastases. Nuclear expression of RYBP was not observed in 11 patients with patient-matched liver metastases. In all adjacent normal liver tissue samples, RYBP was moderately expressed in the cytoplasm (Fig. S1B). In nine of the 11 liver metastases, RYBP was also expressed in the cytoplasm, with a lower staining intensity than in adjacent normal tissue.

Table III. Association between p53 expression and clinicopathological features of patients with colorectal cancer.

Characteristic	p53		P-value
	Wild-type (n=70)	Mutant (n=70)	
Median age, years (range)	71 (30-91)	68 (27-91)	0.1768
Sex, female/male	26/44	23/47	0.5949
Primary lesion site, colon/rectum	43/27	39/31	0.4924
Primary lesion site, right colon/left colon/rectum	8/35/27	4/35/31	0.4415
CEA, <5/≥5 ng/ml	44/26	39/31	0.3895
CA19-9, <37/≥37 U/ml	57/13	57/13	1.0000
Postoperative chemotherapy, no/yes	50/20	45/25	0.3652
T stage[a], 1-3/4	54/16	51/19	0.5580
Lymph node metastasis, no/yes	45/25	39/31	0.3885
Ly, 0/1-3	29/41	32/38	0.6091
V, 0/1-3	31/39	28/42	0.6076
Degree of differentiation, pap or tub/por or muc	68/1	67/3	0.3063
KRAS, wild-type/mutant	7/11	9/18	0.7034
BRAF, wild-type/mutant	1/17	1/26	0.7699
Distant metastasis, no/yes	51/19	50/20	0.8505
Recurrence after primary surgery, no/yes	51/9	50/9	0.9691
Expression of RYBP, high/low	38/32	38/32	1.0000

[a]Union for International Cancer Control 8th edition. RYBP, ring1 and YY-1 binding protein; CRC, colorectal cancer; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; pap, papillary adenocarcinoma; tub, tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma; Ly, lymphatics invasion; V, Vascular invasion.

RYBP regulates tumour cell proliferation in TP53 wt cell lines. As the aforementioned data indicated that RYBP may have a tumour-suppressive role in CRC, *in vitro* experiments were performed to elucidate the molecular mechanisms by which RYBP regulates behaviour of CRC cells. Western blot analyses showed that the expression of both RYBP and p53 was high in HCT116 cells (*TP53*$^{wt/wt}$), whereas expression was low in SW48 cells. However, no association between RYBP and p53 expression was observed in cell lines with *TP53* mutation, such as DLD-1, HT29, COLO201 and SW620 (Fig. 2A). To evaluate the effects of RYBP on *TP53* wt colon cancer cells, HCT116 (*TP53*$^{wt/wt}$) and SW48 cells and *TP53*-mutant DLD-1 and *TP53*-null HCT116 (*TP53*$^{-/-}$) cells were used as the control groups for subsequent experiments.

To verify the function of RYBP, cell proliferation assay was performed following knockdown of RYBP using siRNAs and overexpression of RYBP using pcDNA3.1-RYBP plasmid. RYBP knockdown significantly increased proliferation of HCT116 (*TP53*$^{wt/wt}$) cells. However, knockdown did not alter the proliferation of SW48 cells, in which RYBP expression was low, and in *TP53*-mutant DLD-1 and *TP53*-null HCT116 (*TP53*$^{-/-}$) cells (Fig. 2B). Overexpression of RYBP significantly decreased proliferation of HCT116 (*TP53*$^{wt/wt}$) and SW48, but not *TP53*-mutant DLD-1 and *TP53*-null HCT116 (*TP53*$^{-/-}$) cells (Fig. 2C). These results suggested that RYBP may regulate tumour cell proliferation via a p53-associated pathway.

RYBP induces p53-associated cell cycle arrest and apoptosis. To elucidate the tumour-suppressive mechanisms associated with p53, cell cycle and apoptosis assays were performed. In HCT116 cells (*TP53*$^{wt/wt}$), RYBP knockdown significantly decreased the number of cells in G1/0 phase and increased the number of cells in S phase, whereas RYBP overexpression significantly increased the number of cells in G1/0 and decreased the number of cells in S phase (Fig. 3A). However, the number of HCT116 (*TP53*$^{-/-}$) cells in each cell cycle phase was not affected by modulation of RYBP expression (Fig. S2A). This also indicated that RYBP did not directly damage DNA. Western blot analysis was performed to evaluate expression of p21, a protein downstream of p53, and cyclin D1, a cell cycle G1-S checkpoint regulator (17). In HCT116 (*TP53*$^{wt/wt}$) cells, RYBP knockdown significantly decreased expression of p53 and p21 but induced cyclin D1 expression. Conversely, RYBP overexpression significantly induced expression of p53 and p21 but decreased cyclin D1 expression. However, neither knockdown nor overexpression had any effect on p53, p21 or cyclin D1 expression in *TP53*-mutant DLD-1 and *TP53*-null HCT116 (*TP53*$^{-/-}$) cells (Fig. 3C). We confirmed that RYBP bands were visible in cells transfected with the empty vector in preliminary experiments (Fig. S1C). Protein expression normalised to β-actin is shown in Fig. S3A-C. Corresponding β-actin from same membrane is shown in Fig. S4A and B.

In the apoptosis assay, knockdown of RYBP significantly decreased the number of HCT116 (*TP53*$^{wt/wt}$) cells in early apoptosis, whereas RYBP overexpression significantly

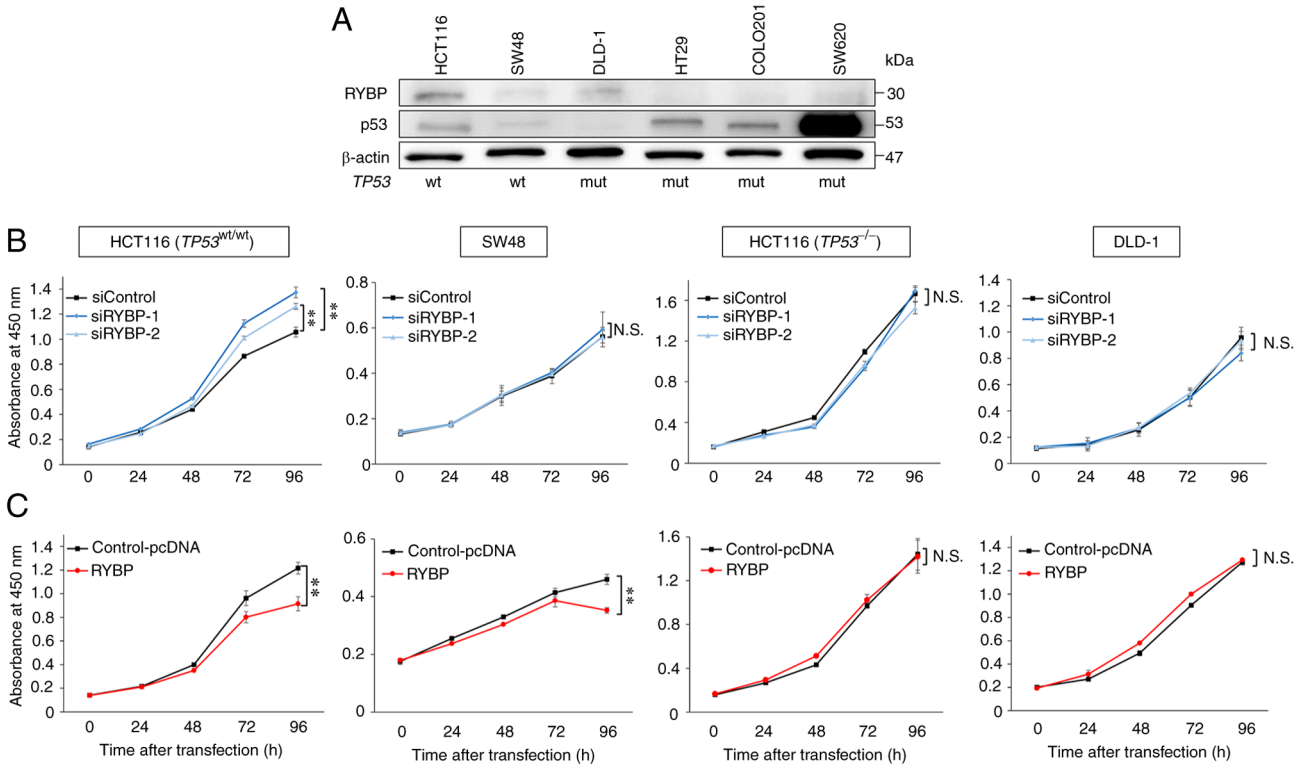


Figure 2. Expression of RYBP in CRC cell lines and cell proliferation. (A) Western blot analysis of RYBP and p53 expression in human CRC cell lines and TP53 genotype. Proliferation assay following (B) siRNA and (C) pcDNA transfection into CRC cells (n=3). Experiments were repeated three times. Data are presented as the mean ± SD. **P<0.01. N.S., not significant; RYBP, RYBP, ring1 and YY-1 binding protein; CRC, colorectal cancer; si, small interfering; mut, mutant; wt, wild-type.

increased the number of apoptotic cells (Fig. 3B). However, the number of apoptotic HCT116 (*TP53*$^{-/-}$) cells was not affected by modulation of RYBP expression (Fig. S2B). Western blot analysis revealed that knockdown of RYBP significantly reduced expression of Bax, a p53-related pro-apoptotic protein, and conversely, RYBP overexpression induced Bax expression (Figs. 3C and S3D).

RYBP enhances oxaliplatin sensitivity by regulating p53-mediated cell cycle arrest. To evaluate the effect of RYBP on oxaliplatin sensitivity, cell viability assay was used to calculate IC_{50} value. In HCT116 (*TP53*$^{wt/wt}$) cells, RYBP knockdown significantly increased viability and the IC_{50} value of oxaliplatin was significantly higher in cells treated with siRNA-1 and siRNA-2 than in the control (Fig. 4A). By contrast, RYBP overexpression decreased cell viability and the IC_{50} was significantly lower in cells treated with pcDNA-RYBP than in the control (Fig. 4B). Low-dose (4.0 μM)oxaliplatin increased the number of cells in G1 phase and decreased the number of cells in the S phase. Higher doses (10 μM) of oxaliplatin further decreased the number of cells in S phase, indicating that disruption of DNA replication leads to anti-tumour effects (Fig. 4C). The results from the cell cycle assay following treatment of cells with 10 μM oxaliplatin showed that RYBP knockdown significantly increased the number of cells in the S phase, whereas RYBP overexpression further decreased the number of cells in S phase (Fig. 4D). These data suggested that RYBP enhanced the sensitivity of CRC cells to oxaliplatin.

Discussion

To the best of our knowledge, the present study is the first to demonstrate that RYBP is an inhibitory protein that protects against distant metastases and recurrence following primary tumour resection in CRC. RYBP stabilises p53 by decreasing mouse double minute 2-mediated ubiquitination and increasing p53 activity (18). The present clinical data showed that low RYBP expression was significantly associated with a higher rate of distant metastases and recurrence, which lead to a poor prognosis in patients with CRC. The tumour-suppressive effect of RYBP was observed in patients with *TP53* wt CRC, but not in those with *TP53* mutant CRC. These data indicated that RYBP plays a role in modulating *TP53* activity. The present *in vitro* data support these clinical data. Using CRC cell lines with or without p53 mutations, the present study demonstrated that RYBP has an effect on cell apoptosis and cell cycle progression by regulating the p53/p21 signalling pathway. Moreover, RYBP increased the sensitivity of CRC cells to oxaliplatin, a key therapeutic drug for CRC (19).

RYBP was originally identified as a member of the non-canonical polycomb repressive complex 1 (7). Studies have demonstrated a key role of RYBP in cancer biology: RYBP expression is downregulated in cancer cells and low RYBP expression is associated with poor prognosis (12,13,20-23). The present clinical data showed the tumour-suppressive role of RYBP in CRC, consistent with the aforementioned studies. To explore the molecular mechanisms underlying these clinical data, the present study performed *in vitro* experiments using

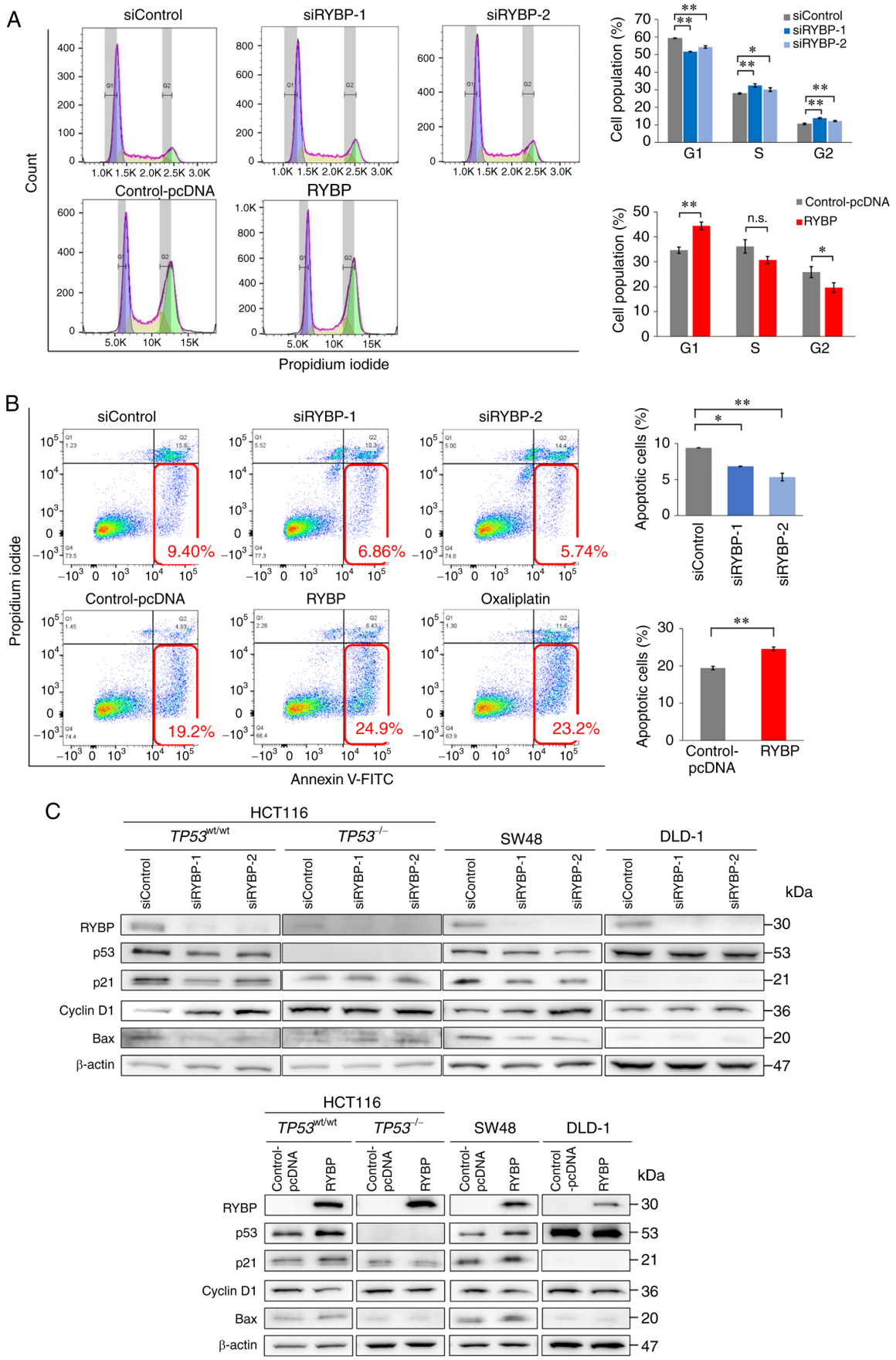
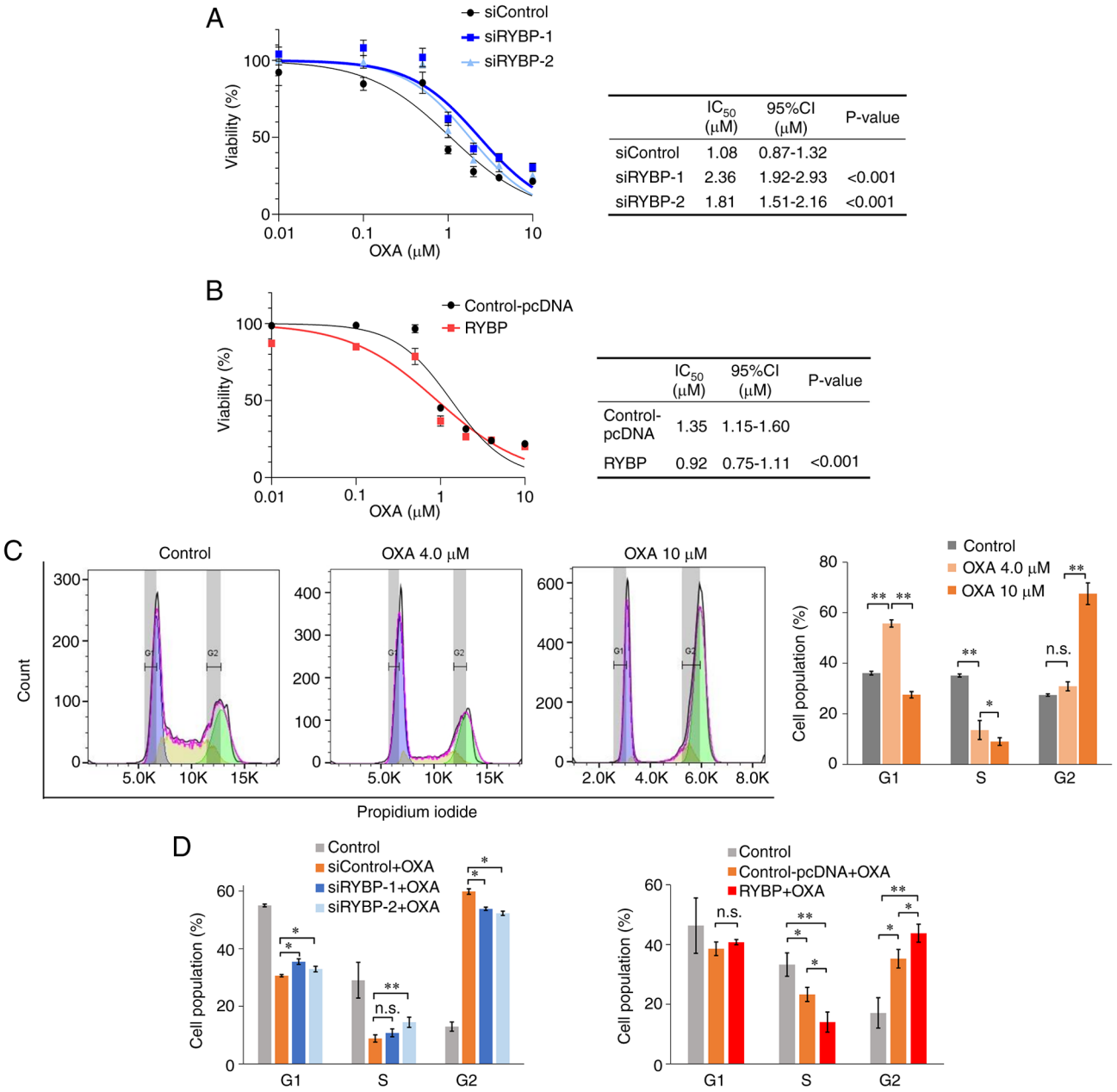


Figure 3. RYBP induces p53-associated cell cycle arrest and apoptosis. (A) Cell cycle distribution in HCT116 (*TP53*$^{wt/wt}$) cells. (B) Distribution of apoptotic cells in HCT116 (*TP53*$^{wt/wt}$) and early apoptotic cells (red box). (C) Levels of RYBP, p53, p21, cyclin D1 and Bax protein detected using western blot analysis after knockdown and overexpression of RYBP (n=3). Some bands were obtained from different membranes with the same cell extract and same loading mass. The experiments were repeated three times. Data are presented as the mean ± SD. *P<0.05, **P<0.01. n.s., not significant; RYBP, RYBP, ring1 and YY-1 binding protein; si, small interfering; wt, wild-type.



	IC_{50} (μM)	95%CI (μM)	P-value
siControl	1.08	0.87-1.32	
siRYBP-1	2.36	1.92-2.93	<0.001
siRYBP-2	1.81	1.51-2.16	<0.001

	IC_{50} (μM)	95%CI (μM)	P-value
Control-pcDNA	1.35	1.15-1.60	
RYBP	0.92	0.75-1.11	<0.001

Figure 4. RYBP enhances OXA sensitivity by regulating p53-mediated cell cycle arrest. Effect of OXA on HCT116 (*TP53*$^{wt/wt}$) cells and IC50 following RYBP (A) knockdown and (B) overexpression (n=6). (C) Cell cycle distribution of HCT116 (*TP53*$^{wt/wt}$) cells after treatment with 0, 4 and 10 μM OXA and (D) 10 μM OXA with or without knockdown or overexpression of RYBP (n=3). Data are presented as the mean ± SD. *P<0.05, **P<0.01. n.s., not significant; CI, confidence interval; RYBP, RYBP, ring1 and YY-1 binding protein; IC_{50}, half-maximal inhibitory concentration; si, small interfering; OXA, oxaliplatin.

CRC cell lines with and without *TP53* mutation. A novel finding of the present study is that RYBP induced apoptosis and G1/S cell cycle arrest by regulating the p53/p21 signalling pathway in *TP53* wt CRC. As a downstream factor of p53, p21 serves a critical role in cell cycle regulation (16,24). DNA damage activates the p53/p21 pathway and induced p21 binds to cyclin D1/cyclin-dependent kinase 4 (CDK4) and cyclin E/CDK2 complexes at the G1 phase checkpoint (17). The binding of p21 to cyclin D1 prevents nuclear transport of the complex, thereby inducing the nuclear accumulation of cyclin D1 and inhibiting its phosphorylation (24). Here, expression of p-cyclin D1 was not changed by knockdown or overexpression of RYBP with or without *TP53* mutation. Since p-cyclin D1 undergoes rapid proteasomal degradation, it is difficult to determine its protein expression by western blotting (25). Independent of the p53/p21 pathway, it is known that p53 directly activates the pro-apoptotic Bcl-2 protein Bax (26). The present data indicated that RYBP-induced p21 suppressed endogenous cyclin D1 expression and lead to cell cycle arrest at G1 phase, resulting in suppression of tumour growth. Additionally, the present data indicated that RYBP-induced Bax upregulation may induce the endogenous apoptotic pathway in CRC.

Although RYBP did not regulate YY-1 expression directly in the present study, previous studies have reported the significant interaction between RYBP and YY-1 (10,11). YY-1 may be associated with the p53 signalling pathway. Our recent study demonstrated downregulated expression of p53 pathway-related genes, such as *CDK2*, Damage Specific

DNA Binding Protein 2, p53 apoptosis effector related to PMP22) and methyl malonyl aciduria cobalamin deficiency B type (*MMAB*), following YY-1 knockdown in colon cancer cell lines using microarray and gene enrichment analyses (4). These data suggest that YY-1 modulates the p53 signalling pathway upstream of YY-1.

In the present study, nine of 11 metastatic tumours did not show nuclear expression of RYBP, although nuclear expression was predominantly observed in primary CRC. The clinical significance of this differential expression pattern is not clear owing to the small sample size. Regarding the subcellular localisation of RYBP, previous studies demonstrated that RYBP predominantly interacts with DED-containing DNA-binding protein (27) or apoptin (28). However, RYBP is also reported to promote the formation of the death-inducing signalling complex in the cytoplasm and induce apoptosis of tumour cells by activating the p53 signalling pathway (29). Our previous study demonstrated that the expression pattern of the critical transcription factor differs between primary and metastatic tumours during tumour progression (5). Similarly, previous studies have demonstrated that the expression pattern of PD-L1 in non-small cell lung cancer (30) and Ki-67 and cyclin D1 in colon cancer (31) differ between primary tumours and matched liver metastases. This may be explained by 'intratumour heterogeneity in space and time' and 'actionable mutation', as proposed by Swanton (32). Tumours actively develop, resulting in metastatic lesions that may be genetically distinct. This tumour heterogeneity facilitates resistance to systemic chemotherapy (1,33). Taken together, the present results suggested that the expression pattern of RYBP may be altered during tumour progression, and this alteration may impact tumour biology. Moreover, RYBP may have different functions depending on the host organ and/or subcellular localisation, even in the same cancer type. These hypotheses need to be tested in future.

Although p53 IHC is used as a surrogate for *TP53* mutations, its accuracy has not been established. Mutant p53 can generally be identified as being overexpressed by immunostaining because of its delayed degradation time and accumulation in the nucleus. By contrast, the absence of p53 in tumour cells indicate a loss-of-function (LOF) mutation in *TP53*. However, no detectable p53 could also indicate *TP53* wt, because wt p53 has a short half-life and is rarely detected by immunostaining, meaning cells with no immunostaining of p53 are considered a mixture of both genotypes (34,35). In a previous study using next-generation sequencing (NGS), 53% of cells with no expression of p53 had LOF mutations and the rest had wt *TP53* (36). Therefore, it is difficult to divide cells into those with LOF mutation and wt *TP53* using IHC only. More accurate assessment of p53 expression using NGS should be conducted in future studies.

Notably, the present data demonstrated that RYBP was associated with chemosensitivity in CRC cells. For advanced-stage CRC, particularly with distant metastases, multidisciplinary treatment in combination with surgical resection and systemic chemotherapy is needed to achieve a cure or long-term survival. An optimal response to systemic chemotherapy is key to maximising the benefits of multidisciplinary treatment strategies. During treatment with successive regimens of chemotherapy, the sensitivity for anticancer drugs may change and tumour cells could become drug resistant by acquiring an embryonic-like and quiescent state epigenetically (37,38). In addition to oxaliplatin, overexpression of RYBP has been shown to enhance sensitivity to cisplatin in hepatocellular carcinoma and anaplastic thyroid cancer (12,21). In addition, *TP53* status has been reported to affect the sensitivity of not only CRC but also esophageal, breast and numerous other types of cancer to chemotherapeutic drugs (39-41). To the best of our knowledge, there are no reports showing the effect of RYBP on 5-fluorouracil or irinotecan, key drugs in the treatment of CRC. However, both drugs induce anticancer effects by inhibiting DNA replication, and it is possible that RYBP enhances their efficacy via the cell cycle and apoptosis (42,43). Therefore, modulation of RYBP expression and RYBP/p53 pathway signalling may be a new target to increase chemosensitivity of oxaliplatin (and other drugs), which could lead to improved clinical outcomes in CRC.

The present data demonstrate a significant association between RYBP and long-term outcome. RYBP may be a useful prognostic predictor in patients with CRC. Moreover, RYBP may be a potential candidate as an effective modulator of systemic chemotherapy for CRC (and other types of cancers).

Accumulating evidence suggests that p53 regulates innate and adaptive immune responses (44,45). Inflammation is involved in the initiation and development of CRC (46). Elevated levels of cytokines such as tumour necrosis factor-α and chemokines such as CXC chemokine ligand 1 and 2 in the serum of patients with CRC are associated with cancer development and progression (47). Therefore, the RYBP/p53 pathway is involved in tumour immunity and chronic inflammation.

The present study has limitations. First, the background of patients was heterogeneous because all data were collected retrospectively. Several prognostic factors such as T stage, degree of differentiation, lymph node metastasis and tumour marker were not randomised for analyses of long-term outcomes. Second, *in vitro* experiments only evaluated RYBP in primary CRC. However, clinical data suggested that its function at metastatic sites may be altered by different pathways and studies in other experimental systems, including *in vivo* experiments, are warranted. Third, the present study evaluated cell proliferation *in vitro* but not ongoing DNA synthesis, which is typically assessed using a BrdU incorporation assay. To evaluate the effect of RYBP on cell proliferation through its regulation of the cell cycle, BrdU assay should be performed. Lastly, in experiments using oxaliplatin, although no significant effect of RYBP was observed on the cell cycle following treatment with 4 μM oxaliplatin (Fig. S2C), effects may vary depending on exposure time and timing of administration, and further investigation is warranted. In conclusion, high RYBP expression in CRC cells was an independent predictor of improved prognosis in patients with CRC. The tumour-suppressive role of RYBP was associated with cell cycle arrest, apoptosis and increased oxaliplatin sensitivity. Further studies are warranted to explore the detailed molecular mechanisms by which RYBP is regulated and to identify factors that enhance RYBP expression in CRC,

which could lead to a novel, effective and safe therapeutic approach for CRC.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

TM and NS designed and performed the experiments. TT, GO, SK, ST, MO and HM made substantial contributions to conception and design of the present study and analysis and interpretation of data. MO and HM confirm the authenticity of all the raw data. TM wrote the manuscript. NS, MO and HM edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All study participants provided written informed consent to participate. The study was approved by the Ethics Committees of the Department of General Surgery, Chiba University Hospital (Chiba, Japan; approval number: M10101). The present study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Written informed consent for publication of clinical details and/or clinical images was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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