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Comprehensive molecular exploration identified promoter DNA methylation of the *CRBP1* gene as a determinant of radiation sensitivity in rectal cancer

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Background: Neoadjuvant chemoradiotherapy (NCRT) for advanced rectal cancer (RC) is a well-evidenced therapy; however, some RC patients have no therapeutic response. Patient selection for NCRT so that non-responsive patients are excluded has been subjective. To date, no molecular markers indicating radiation sensitivity have been reported.

Methods: We irradiated six colorectal cancer (CRC) cell lines and identified HCT116 cells as radiation-sensitive and HCT15 and DLD-1 cells as radiation resistant. Using a microarray, we selected candidate radiation sensitivity marker genes by choosing genes whose expression was consistent with a radiation-resistant or sensitive cell phenotype.

Results: Among candidate genes, cellular retinol binding protein 1 *(CRBP1)* was of particular interest because it was not only induced in HCT116 cells by tentative 10 Gy radiation treatments, but also its expression was increased in HCT116-derived radiation-resistant cells *vs* parental cells. Forced expression of *CRBP1* decreased the viability of both HCT15 and DLD-1 cells in response to radiation therapy. We also confirmed that *CRBP1* was epigenetically silenced by hypermethylation of its promoter DNA, and that the quantitative methylation value of *CRBP1* significantly correlated with histological response in RC patients with NCRT ($P = 0.031$).

Conclusions: Our study identified *CRBP1* as a radiation-sensitive predictor in RC.

There is accumulated evidence to support multimodal therapy for rectal cancer (RC) for the purpose of reduction of the local recurrence rate. Radiotherapy (RT) and addition of chemotherapy to RT has been reported to decrease local recurrence (Kapiteijn *et al*, 2001; Gérard *et al*, 2006). Neoadjuvant chemoradiotherapy (NCRT) is now broadly performed as the initial treatment for locally advanced RC. Tumour response to NCRT covers a wide spectrum, ranging from no to complete response, where only 8 to 24% of the patients show a complete response (Maas *et al*, 2010). A molecular marker that can predict tumour response to NCRT before the therapy is eagerly anticipated because tumour response to NCRT has been shown to be associated with oncological outcomes (Park *et al*, 2012).

The potential of molecular biomarkers to predict tumour response to chemoradiation has been evaluated in several studies. Kuremsky *et al* (2009) reviewed biomarkers for tumour response to NCRT and identified *TP53*, epidermal growth factor (*EGFR*), thymidylate synthase, *Ki-67*, *p21* and *bax/bcl-2* as such biomarkers. However, none of these genes were considered appropriate for clinical use because most of the study results were controversial. In addition, in recent years, gene expression profiles associated with tumour response to chemoradiotherapy have been reported.

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Akiyoshi *et al* (2012) reviewed those studies and concluded that there were some reported gene expression signatures that were capable of high predictive accuracy, but that the compositions of these signatures differed considerably, with little gene overlap. These controversial results were probably due to intratumoral heterogeneity. Since most of those studies assessed gene expression of biopsy samples, those studies could only analyse only a part of the gene expression status of the tumour. From this point of view, the focus should be on a genetic or epigenetic change rather than on gene expression. *KRAS* mutation is one genetic change that has been broadly analysed. Although a correlation between *KRAS* mutation status and the therapeutic effect of anti-EGFR antibody was reported, only a few studies have reported its correlation with chemoradiotherapy and those results are controversial (Van Cutsem *et al*, 2009; Erben *et al*, 2011; Garcia-Aguilar *et al*, 2011; Russo *et al*, 2014; Lee *et al*, 2015). DNA hypermethylation in a gene promoter region is one epigenetic change that is a common event in cancer. We have found that intratumoral heterogeneity is not a limitation in analysis of DNA methylation of biopsy samples (Ooki *et al*, 2010). Ebert *et al* (2012) reported that hypermethylation of the transcription factor AP-2 epsilon gene *TFAP2E* was seen in 51% of the CRC cases analysed and was significantly associated with nonresponse to chemotherapy. However, only a few studies have analysed the correlation between DNA methylation and sensitivity to RT (Tsang *et al*, 2014; Ha *et al*, 2015).

We performed the present study to identify a molecular marker(s) associated with radiation sensitivity in RC. Here we planned a comprehensive exploration of genes associated with radiation sensitivity in colorectal cancer (CRC) cell lines. We analysed differences in gene expression patterns between radiation-sensitive and -resistant cell lines using an expression microarray, and subsequently identified the cellular retinol binding protein 1 (*CRBP1*) gene as a candidate gene whose expression represented radiation sensitivity. Furthermore, we determined the epigenetic silencing of *CRBP1* through promoter hypermethylation and its clinical role in RT for RC.

MATERIALS AND METHODS

Cell lines. The hepatocellular carcinoma cell line HepG2 and the CRC cell lines HCT116, Colo205, Colo320, and LoVo, were purchased from the RIKEN BioResource Center (Ibaraki, Japan). DLD-1 cells were kindly provided by the Cell Response Center for Biochemical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HCT15 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). DLD-1, HCT116, HCT15, Colo205, and Colo320 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS. LoVo cells were grown in L-15 medium (Gibco) supplemented with 10% FBS. Mycoplasma contamination was not tested because neither we, nor other researchers in our institute, have encountered mycoplasma contamination over the past 4 years.

Rectal cancer tissues. Thirty-three RC tissues and corresponding normal mucosa were obtained from patients described in our previously reported study (Nakamura *et al*, 2014). Briefly, a total dose of 45 Gy of RT was administered to those patients. All RC tissues were obtained from a biopsy sample of the primary cancer before therapy and the corresponding normal mucosa tissues were obtained from resected specimens after NCRT.

Genomic DNA extraction. Genomic DNA was extracted from cell lines using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Formalin-fixed paraffin-embedded tissue was cut into six 10 μm slices before genomic DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the manufacturer's instructions. DNA concentration was quantified by UV spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Total RNA extraction. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer. The integrity of total RNA was verified by analysis of the A260/A280 ratio using NanoDrop ND-1000, and by confirming that the result of a reverse transcriptase–PCR (RT–PCR) for *β-actin* was clearly positive.

Total protein extraction. Cultured cells were collected using trypsin and were lysed in RIPA buffer (Thermo Scientific Wilmington, DE, USA) containing protease inhibitors. Protein concentration was measured using the Bio Photometer (Eppendorf, Hamburg, Germany).

Radiation sensitivity assay. Cells (1×10^6) of each cell line were seeded into 10-cm dishes. On the next day, each cell line was irradiated with a dose of 1 to 4 Gy per day for 5 days. Cells incubated without radiation were used as a control. On day 7, the cells were collected by trypsinisation and the cells were counted using Countess (Invitrogen, Carlsbad, CA, USA). The assay was performed once for each cell line. Radiation sensitivity was evaluated by measuring the ratio of the number of live irradiated cells compared to the number of control cells.

Microarray analysis. Total RNA was extracted from HCT15, DLD-1, and HCT116 cells. Gene profiles were compared using GeneChip 3′ IVT Express Kit microarrays (Affymetrix; Santa Clara, CA, USA) according to the manufacturer's instructions. Genes expressed at high levels in the radiation-sensitive cells and at low levels in the radiation-resistant cells were categorised as radiation sensitivity-related genes. Genes expressed at high levels in the radiation-resistant cells and at low levels in the radiation-sensitive cells were categorised as radiation resistance-related genes.

Establishment of radiation-resistant cells. HCT116 cells (1×10^6 cells) were cultured in 10 cm dishes and irradiated with 3 Gy at 60–70% confluence. On day 7, the cells were trypsynised and 1×10^6 cells were re-seeded in 10 cm dishes and similarly irradiated. This cycle was repeated 20 times, following which the radiation dose totaled 60 Gy (I-HCT116). Concurrently, other HCT116 cells were cultured without radiation and re-seeded once a week (M-HCT116). Evaluation of resistance to radiation was performed using the following protocol. Parental HCT116 (P-HCT116), I-HCT116 and M-HCT116 cells were each seeded into two 10 cm dishes at a density of 1×10^6 cells per dish. One dish was designated as the radiation group and the other dish was used as the control. On the next day, the radiation study groups were irradiated with a dose of 1 Gy per day for 5 days. The cells were counted on day 7. The ratio of the number of cells of the radiation and control groups was calculated. Each assay was performed in triplicate.

Selection of candidate genes related to radiation sensitivity and resistance among heat map genes. To select the optimal candidate genes related to radiation sensitivity, we focused on genes that met the following criteria: (1) increased expression was seen in radiation-sensitive cells after short-term 10 Gy irradiation and expression was not seen in radiation-resistant cells; and (2) expression was further increased after long-term 60 Gy irradiation (Figure 3B). The criteria for selection of candidate genes related to radiation resistance were as follows: increased expression was seen in radiation-resistant cells after short-term 10 Gy irradiation and expression was not seen in radiation-sensitive cells.

Reverse transcriptase–PCR. First strand cDNA was synthesised from RNA using SuperScript III reverse transcriptase (Invitrogen) and Oligo (dT) primers (Invitrogen) as follows. Total RNA (2 μg) was transcribed using Oligo (dT) primers. The sample was heated to 65 °C for 5 min and then quickly chilled on ice. SuperScript III reverse transcriptase (200 units) and reaction buffer was added to the tube and incubated at 50 °C for 30 min and then 70 °C for 15 min. The obtained cDNA was used for both RT–PCR and quantitative real-time RT–PCR (qRT–PCR). RT–PCR was carried out using Platinum Taq DNA Polymerase (Invitrogen) according to the manufacturer's protocol. The PCR conditions were: 5 min at 95 °C followed by the appropriate number of cycles of 95 °C for 1 min, the appropriate annealing temperature for 1 min, and 72 °C for 1 minute, and a subsequent final incubation at 72 °C for 10 min. Quantitative RT–PCR was performed in triplicate using TaqMan probes and iQ Supermix (Biorad, Hercules, CA, USA), and the CFX96 real-time system (Biorad). The PCR conditions were as follows: 3 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The primer sequences and specific PCR conditions for each gene are shown in Supplementary Tables S1 and S2. The mRNA expression value was defined as the quantity of fluorescence intensity derived from amplification of the *CRBP1* gene divided by the fluorescence intensity from amplification of *β-actin*, multiplied by 100.

Plasmid construction for transfection into cell lines. Full-length *CRBP1* cDNA was synthesised from total RNA extracted from HCT116 cells by PCR using Platinum Taq Polymerase and Oligo (dT) primers, and was cloned into the pcDNA3.1 myc-His C expression vector (Invitrogen) using the restriction enzymes *EcoR*I and *Xho*I, and the T4 Ligase. The plasmid sequence was directly sequenced to confirm the fidelity of the *CRBP1* insert. Cells were transfected with this plasmid using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) as per the manufacturer's instructions.

Confirmation of forced expression of *CRBP1* after transfection. The mRNA and protein expression of *CRBP1* after transfection was confirmed using RT–PCR and western blotting, respectively. Total RNA and total protein was extracted 48 h after transfection for further RT–PCR and western blotting.

Western blotting analysis. Total cellular protein (60 μg) was loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrophoresis was performed followed by electroblotting to a PVDF membrane (Invitrogen). The blots were incubated with anti-myc (Invitrogen) and anti *β-actin* (Invitrogen) antibodies that were used as primary antibodies. The blots were developed using Western Breeze (Invitrogen), which contains alkaline phosphatase-conjugated anti-mouse immunoglobulin and a chemiluminescent substrate for alkaline phosphatase. Signals were detected using the luminescent image analyzer ImageQuant LAS 4000 (GE Healthcare, CT, USA).

Cell proliferation assay. Cell proliferation was assayed using the CytoSelect water-soluble tetrazolium salt (WST-1) Cell Proliferation Assay Reagent (Cell Biolabs, San Diego, CA, USA). On day 1, the cells were cultured in a 96-well plate at a density of 1×10^4 cells per plate. On day 2, the cells were transiently transfected with *CRBP1*. On day 3, cell proliferation was evaluated by measuring the optical density (OD) at 450 nm.

Radiation sensitivity assay after cell transfection. We analysed increased cellular radiation sensitivity by the WST-1 assay and cell counting. In the WST-1 assay, on day 1, the cells were seeded in a 96-well plate (1×10^4 cells per plate). On day 2, the cells were transiently transfected with *CRBP1*. The cells were irradiated with a dose of 5 Gy per day on days 3 and 4, and were analysed with the WST-1 assay on day 6. For cell counting, 2×10^5 cells were seeded in a 6-well plate on day 1. On day 2, the cells were transiently transfected with *CRBP1*. The cells were irradiated with a dose of 5 Gy per day on days 3 and 4, and were counted on day 6. Non-radiated but transfected cells were incubated concurrently as controls for both the WST-1 and the cell count assays. The ratio of the absorption of irradiated and non-irradiated cells (control) was calculated.

Bisulfite treatment of genomic DNA. Genomic DNA (2 μg) was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zimo Research, Irvine, CA, USA) following the manufacturer's instructions.

Bisulfite sequence analysis. Bisulfite treated DNA from cell lines was amplified using PCR. Primer sequences for amplification of the *CRBP1* promoter region were designed to recognise DNA alterations caused by bisulfite treatment (Supplementary Table S1). The PCR products were purified using QIAquick Spin (Qiagen) and were either directly sequenced or were inserted into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequence (Invitrogen). Ten clones were analysed for each cell line. We performed direct sequence analysis to screen for the existence of methylation and we used the cloned sequence to confirm the exact status of methylation.

Quantitative methylation-specific PCR (qMSP). For qMSP analysis of *CRBP1*, we performed real-time PCR using iQ Supermix and CFX96 real-time systems. Two micrograms of bisulfite treated samples were loaded into each well. The PCR conditions were as follows: 3 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 62 °C for 30 s, and 72 °C for 30 s. Primer and hybridisation probe sequences are shown in Supplementary Table S1. Bisulfite treated DNA from DLD-1 cells was used as the methylation positive control for construction of a calibration curve on each plate, because cloned sequence analysis showed the cytosines in all of the clones of DLD-1 were almost completely methylated. Bisulfite treated DNA from HepG2 cells was used as the negative control because almost all of the cytosines were unmethylated. All reactions were performed in triplicate. The methylation value TaqMeth V) was defined as the quantity of fluorescence intensity derived from amplification of the *CRBP1* gene divided by the fluorescence intensity derived from amplification of *β-actin*, multiplied by 100. This ratio was used as a measure for the relative level of methylated DNA in samples.

5-Aza-dC and TSA treatment. Cells were seeded in a 10 cm dish on day 0. The cells were then treated every 24 h for 4 days with either 1 or 5 μM 5-Aza-dC (5-aza-2′-deoxycytidine) dissolved in 50% acetic acid or were mock treated with PBS including the same amount of acetic acid. Trichostatin A (TSA; 300 nM; Sigma Aldrich, Inc, St Louis, MO, USA) was added to the medium for the final 24 h. On day 5, the cells were harvested and mRNA was extracted. RT–PCR was performed to confirm the re-expression of silenced genes.

Evaluation of pathological specimens. Tumour responses to NCRT were evaluated by histopathological examination of serial sections of resected specimens after surgery. Responses were evaluated according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (Japanese Society for Cancer of the Colon and Rectum, 2009). This study was conducted in accordance with the Declaration of Helsinki, and all patients signed a consent form approved by the Research Ethics Committee of Kitasato University School of Medicine.

Statistical analysis. Either χ^2 or Fisher exact tests were used for categorical variables. Student's *t*-test was used for continuous variables (cell count and cell proliferation assays). *P*-values < 0.05 were considered to represent statistical significance.

RESULTS

Identification of radiation-sensitive and -resistant CRC cell lines. Initially, we irradiated six CRC cell lines to determine their radiation sensitivity. DLD-1, HCT116, HCT15, LoVo, Colo320 and Colo205 cells were irradiated with various doses (from 1–4 Gy per day) and the cell count was performed after the radiation treatments (Figure 1A). With the dose of 1 Gy per day, the highest percentage of live cells was found in HCT15 and DLD-1 cells (86% and 73%, respectively), while the lowest percentage was found in HCT116 cells (27%; Figure 1B). This trend in the percentage of live cells per cell line was preserved with radiation doses of 2, 3 and 4 Gy per day although the difference in the number of live cells between the cell lines became smaller than that at 1 Gy per day presumably due to the toxic effect of the radiation treatments. Based on these results, we designated HCT15 and DLD-1 cells as radiation-resistant and HCT116 cells as radiation-sensitive for the purposes of this study.

Establishing radiation-resistant cells by repeated irradiation. We investigated if radiation-sensitive cells could be converted into radiation-resistant cells by continuous irradiation. HCT116 cells were therefore repeatedly irradiated with a dose of 3 Gy per week, to a final total dose of 60 Gy as shown in Figure 1C. Subsequently, we evaluated if these I-HCT116 cells had indeed became resistant to radiation treatment by comparison of the cell number of I-HCT116, P-HCT116 and M-HCT116 after 5 Gy radiation treatments (Figure 1D). The survival of I-HCT116 cells was significantly higher than that of either P-HCT116 ($P = 0.005$) or M-HCT 116 ($P = 0.003$) cells. We, therefore, concluded that the I-HCT116 cells had become resistant to radiation treatment over time.

Comprehensive exploration of genes related to radiation sensitivity. To identify candidate genes underlying radiation sensitivity, gene expression of both radiation-sensitive and radiation-resistant cells was analysed using expression microarrays. Candidate genes were initially restricted to those abundantly expressed in each cell line (defined as 'present expression' in the microarray), resulting in 14 235 candidate genes in the radiation-sensitive cell line HCT116 and 13 288 candidate genes in the radiation-resistant cell lines HCT15 and DLD-1. Genes that showed a high sensitive cells/resistant cells expression level ratio in HCT116 cells were then chosen, of which we selected the top 40 genes. Additionally, genes that showed a high resistant cells/sensitive cells expression level ratio were also chosen, of which we chose 26 genes that were common to both resistant cell lines (Figure 2). Heat maps of the differentially expressed genes between radiation-resistant and radiation-sensitive cell lines are shown in Figure 3A. The microarray results for all candidate genes were confirmed using RT–PCR (Figure 3B).

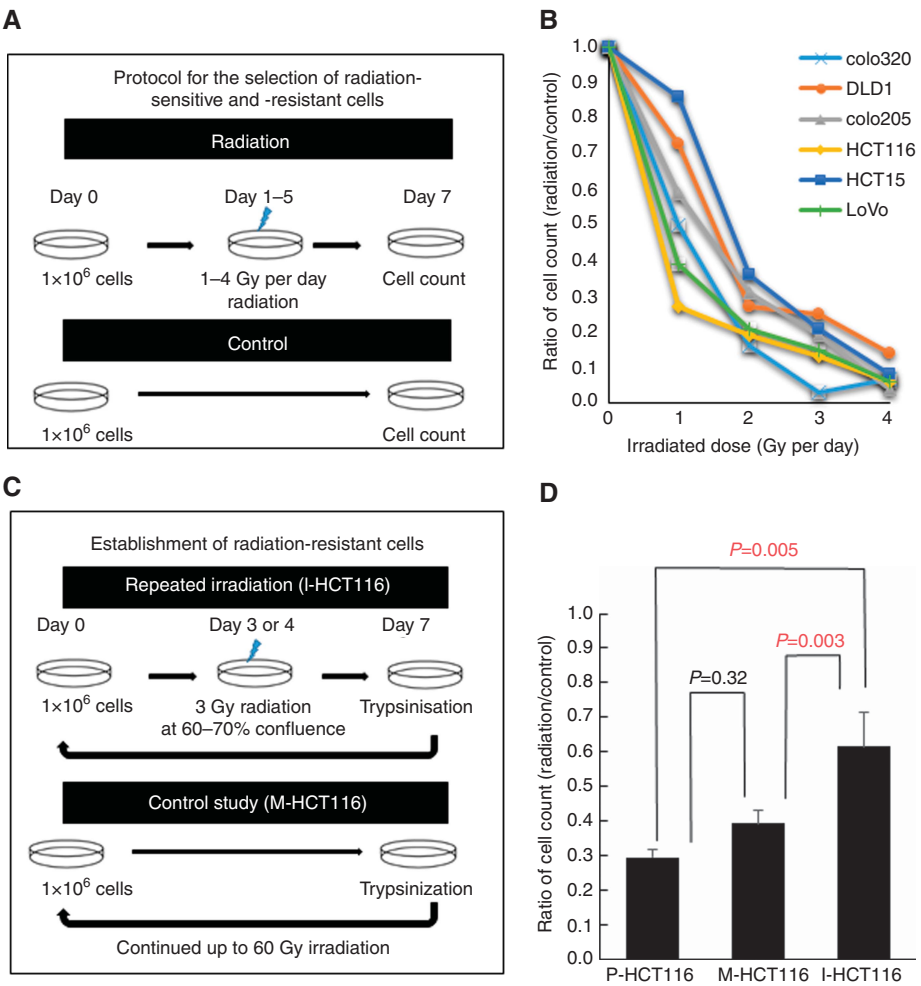


Figure 1. Radiation sensitivity assay and establishment of radiation-resistant cells. (**A**) The protocol for selection of radiation-sensitive and resistant cells. (**B**) The ratio of the cell number in each cell line after to before radiation. (**C**) The protocol for establishment of radiation-resistant HCT116 cells. (**D**) Cell count after 5 Gy radiation for confirmation of the acquisition of radiation resistance following exposure of HCT116 cells to continuous RT to a total of 60 Gy.

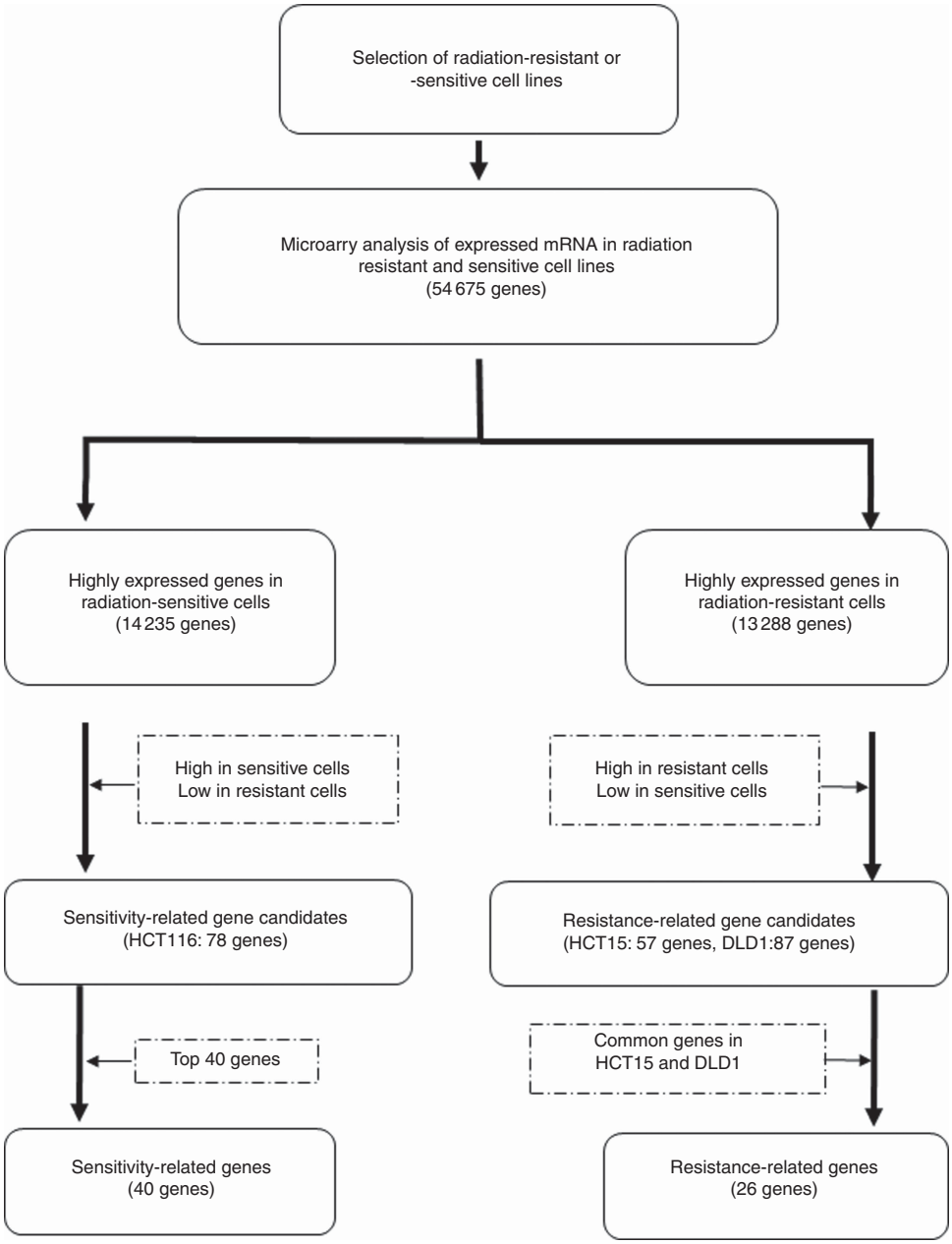


Figure 2. Schematic outline of the definition of radiation sensitivity and resistance-related genes in microarray analysis.

Selection of candidate genes related to radiation sensitivity among heat map genes. Three genes (*CRBP1*, *STC2* and *SLCO3A1*) matched our criteria for involvement in cellular radiation sensitivity (Figure 3C and D). Of these genes, we focused on *CRBP1* because several other studies have reported *CRBP1* as a tumour suppressor gene (TSG) candidate. Increased expression of *CRBP1* in cell lines was validated using qRT–PCR (Figure 3E). *CRBP1* expression increased in HCT116 cells after tentative 10 Gy irradiation, but was counterintuitively also increased in the I-HCT116 cells. *CRBP1* expression was not detected in HCT15 or DLD-1 cells, even after the tentative irradiation. We also tried to identify genes related to radiation-resistance by using the converse criteria to those used for the radiation-sensitive genes, but none of the genes matched these criteria. Therefore, we only focused on the radiation-sensitive candidate genes in the present study.

Forced expression of *CRBP1* increased radiation sensitivity in the radiation-resistant cell lines. To determine the functional relevance of *CRBP1* for radiation sensitivity, we first analysed *CRBP1* expression in the cell lines before and after short-term radiation using qRT–PCR. The basal expression of *CRBP1* tended to increase as cellular radiation sensitivity increased. Radiation-induced increased expression of *CRBP1* was detected in Colo205 and HCT116 cells (Figure 4A).

We then transiently transfected a plasmid vector encoding full-length *CRBP1* under the control of the cytomegalovirus promoter region into the radiation-resistant CRC cell lines (HCT15 and DLD-1; Figure 4B). We first assessed the effect of *CRBP1* transfection on cell viability using the WST-1 assay. A significant decrease in DLD-1 and HCT15 cell viability was observed after *CRBP1* transfection (Figure 4C; $P<0.0001$ and $P=0.0004$, respectively, *vs* vector-transfected cells). Next, we similarly assayed the viability of *CRBP1*-transfected DLD-1 and HCT15 cells after radiation. A significant decrease in cell viability (radiation *vs* control) was observed in the two cell types (Figure 4D; $P=0.0291$ and $P=0.0312$, respectively).

A significant decrease in cell number ratio (radiation *vs* control) was observed only in DLD-1 cells ($P = 0.0071$; Figure 4E). On the basis of these results, we considered that *CRBP1* acted as a TSG, and that its forced expression resulted in the acquisition of a phenotype of RT sensitivity by DLD-1 and HCT15 cells (Figure 4F).

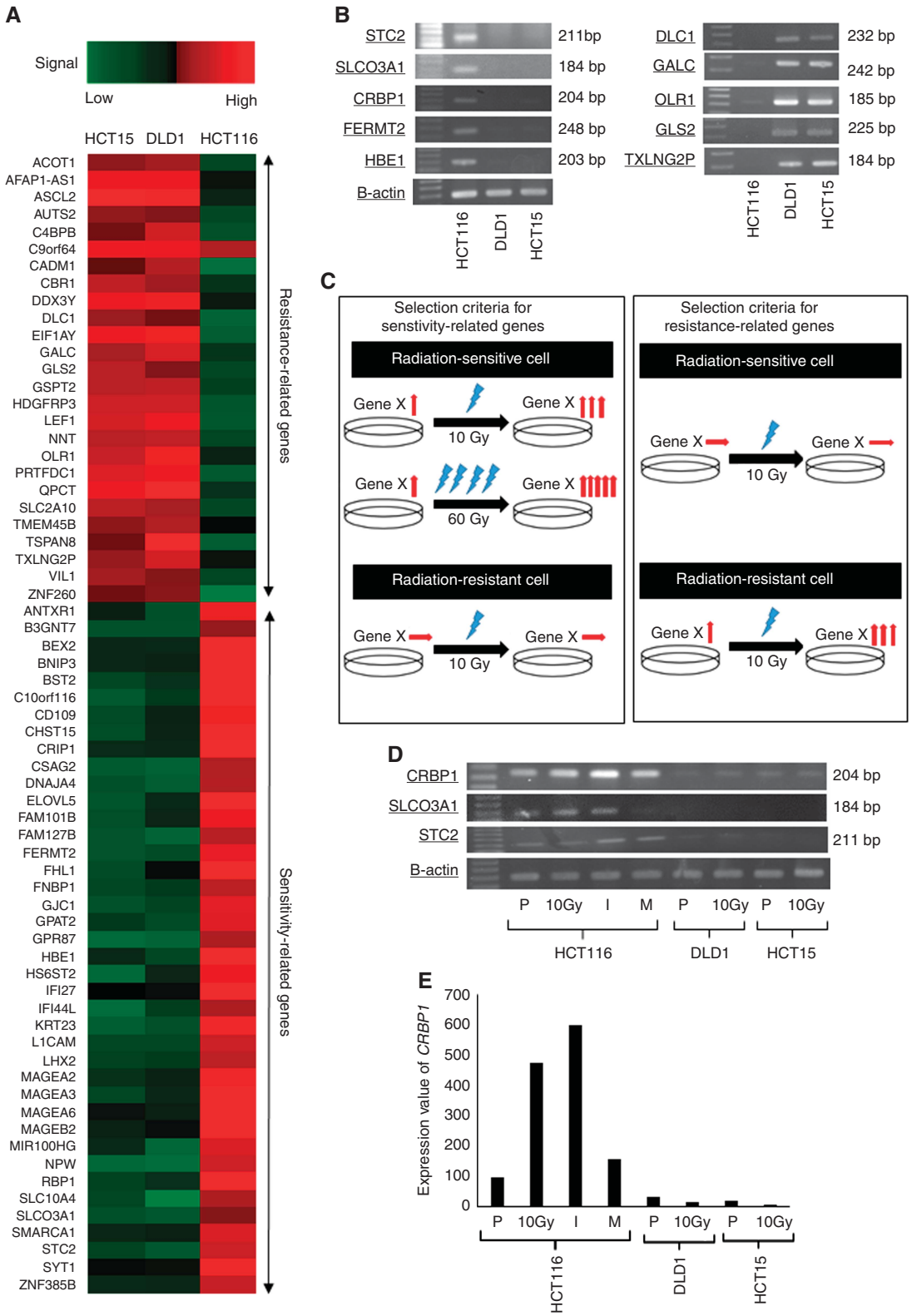


Figure 3. Selection of candidate genes related to radiation sensitivity among the heat map genes. (**A**) Heat map of Affymetrix GeneChip gene expression microarray in radiation-resistant and -sensitive CRC cells. Red coloured bars indicate upregulated genes and green coloured bars indicate downregulated genes. (**B**) Relative results of RT–PCR analysis, which was performed to confirm microarray analysis results. (**C**) The selection criteria for sensitivity-related and resistance-related genes. (**D**) The results of RT–PCR for the selection of sensitivity-related genes. P, Parental cell; 10 Gy, after 10 Gy radiation; I, I-HCT116; M, M-HCT116 (Figure 1C) (**E**) The results of quantitative RT–PCR according to *CRBP1* expression.

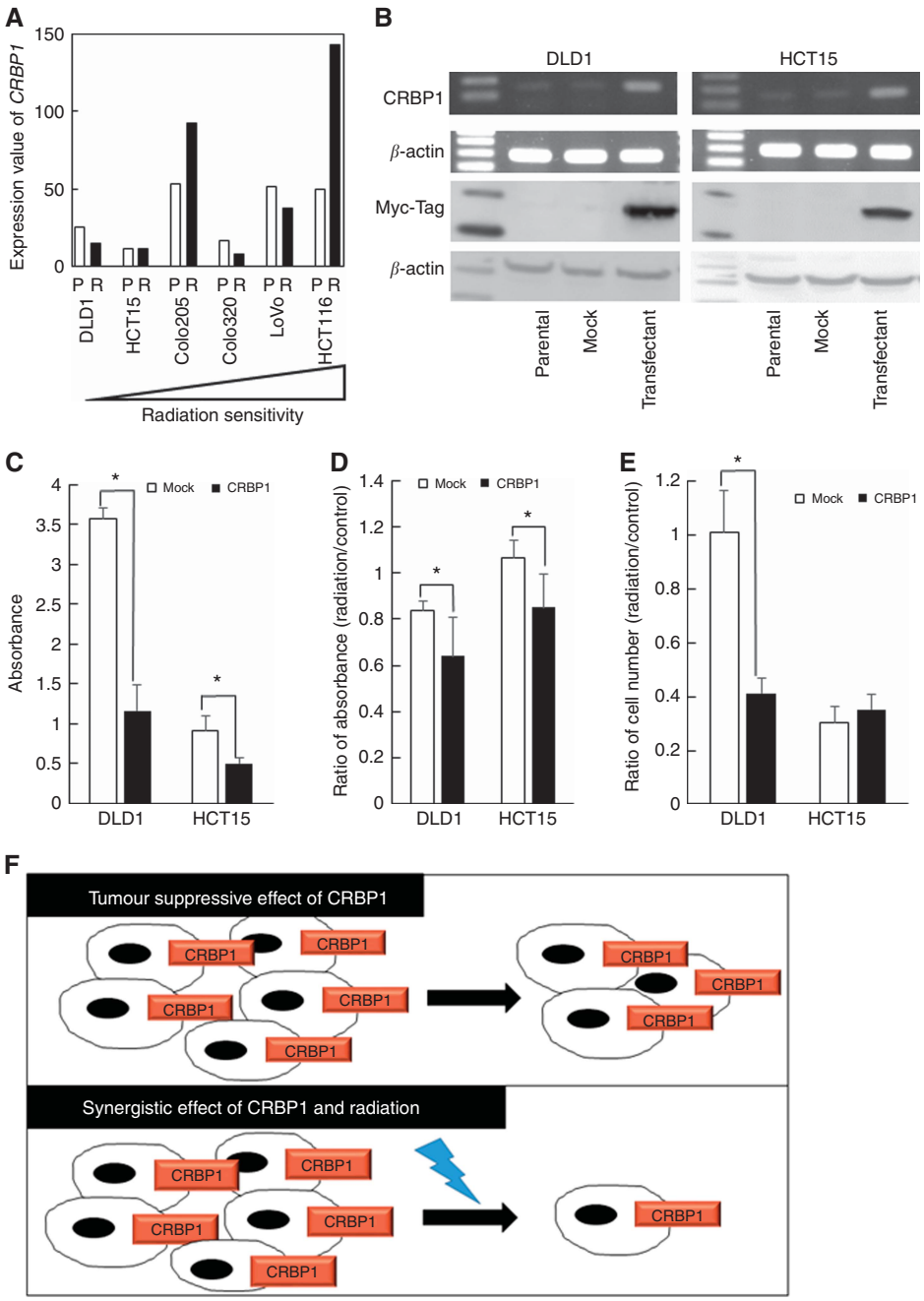
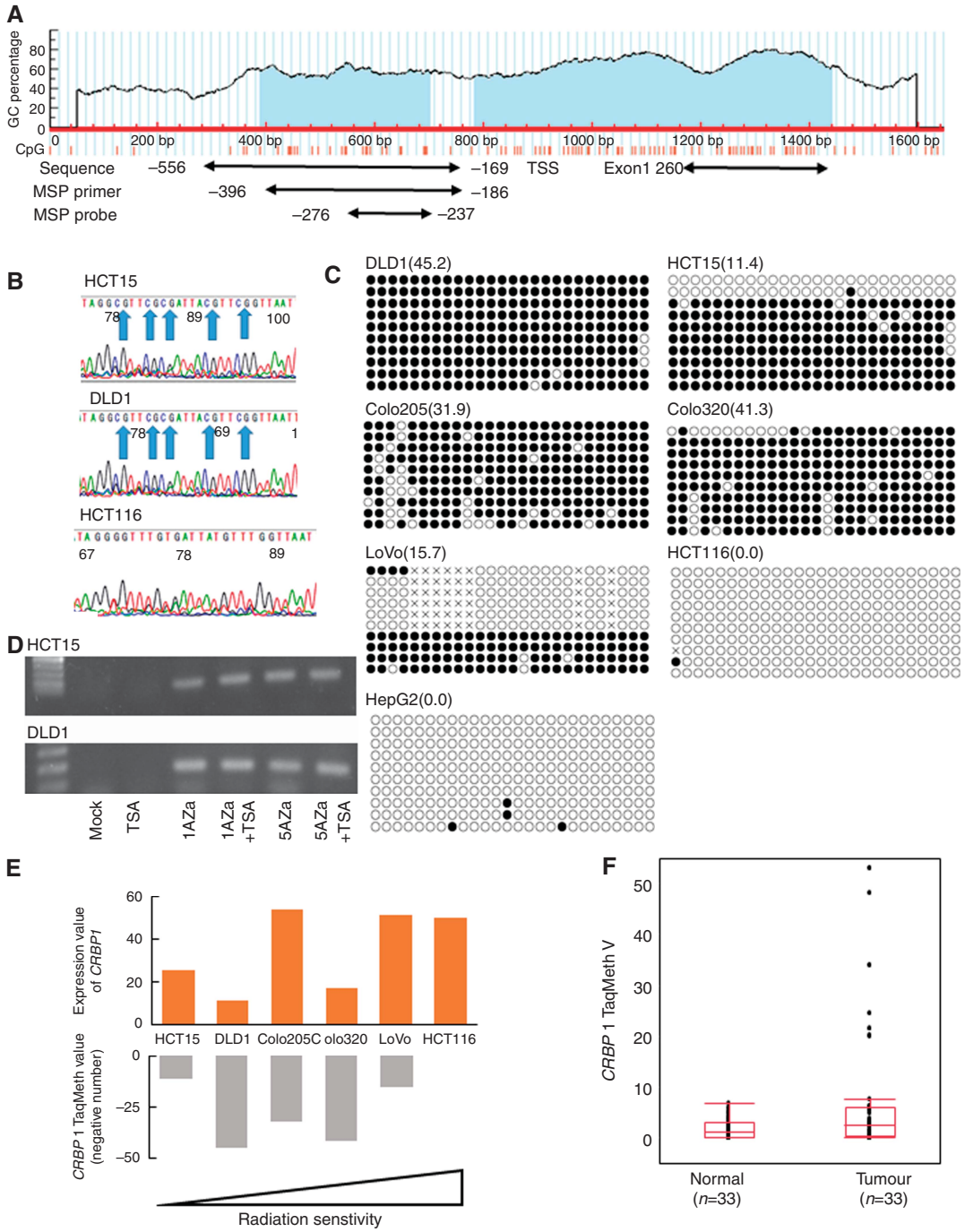


Figure 4. Changes of radiation sensitivity by the forced expression of *CRBP1* gene. (**A**) The result of quantitative RT–PCR analysis of *CRBP1* expression in CRC cell lines. P, Parental; R, after 10 Gy radiation. (**B**) RT–PCR and Western blotting to confirm *CRBP1* expression in *CRBP1*-transfected HCT15 and DLD-1 cells. (**C**) Cell proliferation assay after *CRBP1* transfection. (**D**) Cell proliferation assay after radiation of transfected CRC cell lines. (**E**) Cell counts after radiation of transfected CRC cell lines. (**F**) Scheme of *CRBP1* function.

DNA methylation of the *CRBP1* promoter in the CRC cell lines. We next analysed the methylation pattern of the *CRBP1* gene in the CRC cell lines. For this purpose, we designed a unique primer for bisulfite sequencing and qMSP using 'Meth primer' software (Li and Dahiya, 2002). Two regions within the *CRBP1* promoter region were indicated as CpG islands. One region is situated upstream of the transcription start site (TSS), and the other region is situated from upstream of the TSS to the end of exon 1 (Figure 5A). We focused on the former region in the present study. First, we performed direct bisulfite sequencing of this *CRBP1* promoter region in HCT15, DLD-1, and HCT116 cells (Figure 5B). HCT15 and DLD-1 cells harboured densely methylated CpG islands whereas *CRBP1* genomic DNA in HCT116 cells was not methylated. The methylation patterns of the *CRBP1* promoter region in the 6 CRC cell lines were then analysed by bisulfite sequencing of cloned PCR products (Figure 5C). Finally, we quantified the *CRBP1* gene methylation level of each CRC cell line using qMSP. Radiation-resistant cells displayed a significantly higher TaqMeth V than radiation-sensitive cells (Figure 5E). Furthermore, re-expression of *CRBP1* was observed in HCT15 and DLD-1 cells following their treatment with demethylating agents (Figure 5D). A close association between the TaqMeth V and suppressed *CRBP1* mRNA expression was found in CRC cell lines (Figure 5E).



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*CRBP*1 TaqMeth V	Therapeutic responce of tumour		*P*-value
	Grade 1–2	Grade 3	
TaqMeth value<2.79	12	7	0.031
TaqMeth value>2.79	13	1	

Figure 5. Analysis of promoter hypermethylation of *CRBP1* gene in CRC cell lines and clinical samples. (**A**) Schematic diagram of CpG islands within the *CRBP1* promoter region. TSS; translation start site. (**B**) Representative results of bisulfite sequencing of HCT15, DLD-1, and HCT116. The boxed arrow indicates methylated cytosine. (**C**) Methylation status of 26 individual CpG sites (columns) of 10 cloned PCR products (rows) within the *CRBP1* promoter region by bisulfite sequencing. Open and filled circles, unmethylated and methylated CpG sites, respectively. Numbers in parenthesis denote TaqMeth V. (**D**) The result of RT–PCR analysis of *CRBP1* after demethylation treatments in HCT15 and DLD1 cells. TSA, trichostatin A; Aza, 5-Aza-dC. (**E**) Correlation between expression and methylation status in CRC cell lines. (**F**) Distribution of the *CRBP1* TaqMeth V in tumour tissue and corresponding normal mucosa. (**G**) Correlation between therapeutic response of 33 RC tissues and the TaqMeth V.

***CRBP1* promoter DNA methylation in preoperative biopsy samples and corresponding normal mucosa of RC patients with NCRT.** We considered that qMSP was the optimal method for high throughput analysis to quantify the *CRBP1* promoter DNA methylation level in patient samples. We therefore analysed 33 biopsy samples of RC patients before NCRT and the 33 corresponding normal mucosa using qMSP. The clinicopathological parameters of all patients analysed are shown in Table 1. Tumour samples showed significantly higher TaqMeth *Vs* than the corresponding normal mucosa ($P = 0.0122$; Figure 5F). Tumour specific *CRBP1* promoter hypermethylation was seen in RC patients. Grade 3 was seen in 8 cases, among which 7 cases showed a low *CRBP1* TaqMeth V, and surprisingly, only one grade 3 patient showed a high TaqMeth V. Of 25 cases with Grade 1–2, 13 patients had a low *CRBP1* TaqMeth V (Figure 5G). There was a significant correlation between the *CRBP1* TaqMeth V and histological response ($P = 0.031$). Taking into consideration the basic experiments reported herein, the patients with a low TaqMeth V were regarded as radiation-sensitive.

DISCUSSION AND CONCLUSION

In our screen, we identified three potential radiation sensitivity-related genes using our criteria: *CRBP1*, *STC2* and *SLCO3A1*. The functional role of *SLCO3A1* has not been studied extensively, although transcripts of *SLCO3A1* were upregulated in pancreatic cancer (Hays *et al*, 2013). Stanniocalcins (STCs) are glycoprotein hormones, and several investigators have reported their role in calcium and phosphate homeostasis (Ishibashi and Imai, 2002; Chang *et al*, 2003). Upregulation of *STC2* has been reported in several cancers (Ieta *et al*, 2009; Kita *et al*, 2011; Arigami *et al*, 2013; Fang *et al*, 2014; Hashemzadeh *et al*, 2014). High levels of *STC2* expression were correlated with worse prognoses of cervical cancer patients after RT (Shen *et al*, 2014). Since both *STC2* and *SLCO3A1* are thought to have oncogenic roles in cancer they were considered inappropriate for further investigation in our study. On the other hand, decreased expression of *CRBP1* or hypermethylation of the *CRBP1* promoter have been reported in multiple cancers in several reports (Esteller *et al*, 2002; Yamashita *et al*, 2002; Jerónimo *et al*, 2004; Kwong *et al*, 2005; Toki *et al*, 2010; Colvin *et al*, 2011; Peralta *et al*, 2012; Mendoza-Rodriguez *et al*, 2013). Analysis of the expression of *CRBP1* in CRC cell lines suggested that it may have a functional role in radiation sensitivity in at least four cell lines. Cell lines with higher basal expression of *CRBP1* (HCT116 and LoVo) tended to be radiation-sensitive, and HCT116 cells could express higher CRBP1 by short-term radiation. Conversely, cell lines with lower basal *CRBP1* expression (HCT15 and DLD-1) were radiation-resistant and neither of these cell lines could induce *CRBP1* expression after short-term radiation. Moreover, enforced *CRBP1* expression reduced DLD-1 and HCT15 cell viability. A reduction in cell viability was also seen even after RT of *CRBP1*-expressing DLD-1 and HCT15 cells. Based on these results, we concluded that *CRBP1* acts as a TSG and strongly contributes to cellular radiation sensitivity (Figure 5F). *CRBP1* contribution to radiation sensitivity might be higher in DLD-1 cells than in HCT15 cells, because the cell number of *CRBP1*-transfected HCT15 cells was not decreased by radiation, while that of *CRBP1*-transfected DLD1 cells was decreased. On the basis of these results it might be expected that the established radiation-resistant cell line (I-HCT116) would express less *CRBP1* than the parental cell, but in fact its *CRBP1* expression was somewhat higher. This finding suggested that there may be a critical pathway related to radiation sensitivity that is regulated by a gene downstream of *CRBP1*, and that this downstream gene was inactivated by a mechanism such as DNA methylation or gene mutation. Connected with this finding, there was one clinical sample of a rectal cancer case that showed grade 3 after NCRT, but in which there was high *CRBP1* promoter DNA methylation. Qualitative or semi-quantitative analyses of *CRBP1* promoter methylation have been reported in several studies (Jerónimo *et al*, 2004; Kwong *et al*, 2005; Toki *et al*, 2010; Peralta *et al*, 2012; Mendoza-Rodriguez *et al*, 2013). In this study, we performed quantitative analysis by using qMSP. There was a clear correlation between the degree of *CRBP1* methylation and *CRBP1* expression in DLD-1 and HCT116 cells, which had distinguishable radiation-sensitivity phenotypes. However, in HCT15 cells, while their *CRBP1* expression was relatively low and their phenotype was radiation-resistant, *CRBP1* methylation was low. We speculate that, in HCT15 cells, silencing of *CRBP1* expression was regulated not by its promoter hypermethylation but by its upstream genes. Although Colo205 cells showed a high *CRBP1* expression level, the *CRBP1* promoter region that we analysed was hypermethylated. This result suggested the existence of other CpG islands in the *CRBP1* promoter whose hypermethylation should be analysed. It was a limitation of our study.

A significant correlation between the TaqMeth V and the therapeutic response of RC was seen in the present study, in which hypermethylation of the *CRBP1* promoter was correlated with a poor therapeutic effect of RT. Of the 14 cases with a high TaqMeth V, 13 cases (93%) were grade 1–2 cases. These 13 cases constituted 52% (13/25) of the grade 1–2 cases. These data indicate that *CRBP1* promoter methylation status could be a good indicator to preoperatively identify radiation-resistant patients. However, we could not predict non-responders to NCRT among these 13 patients. A biomarker that predicts non-responders to NCRT is required since non-responders should not be given NCRT and must be operated on immediately. Another marker for detecting such patients should be explored. It is a limitation of our study.

Table 1. Clinicopathological parameters of all analysed patients

Clinicopathological parameters	Number of patients (%)
Age	
Mean ± s.d.	62.9 ± 9.4
Median (range)	65 (32–78)
Sex	
Male	24 (69.7%)
Female	9 (27.3%)
Location	
Ra	16 (48.5%)
Rb	17 (51.5%)
T factor	
T3	28 (84.8%)
T4	5 (15.2%)
N factor	
N0	23 (69.7%)
N1	9 (27.3%)
N2	1 (3.0%)
Stage	
II	23 (69.7%)
III	10 (30.3%)
Histological grade	
1a	4 (12.1%)
1b	8 (24.2%)
2	13 (39.4%)
3	8 (24.2%)
TaqMeth V	
Mean ± s.d.	7.93 ± 21.3
Median (range)	2.66

Abbreviations: Ra = rectum above pertoneal reflection; Rb = rectum below peritoneal reflection.

In conclusion, to date, there is no good predictor of tumour response to RT. However, our comprehensive molecular exploration identified *CRBP1* as a candidate mediator of cellular radiation sensitivity, and our functional studies of *CRBP1* confirmed that it plays an important role in radiation sensitivity, and that hypermethylation of its promoter region was correlated with resistance to RT. However, *CRBP1* was not completely consistent with radiation sensitivity in all tested cell lines or clinical samples. This present investigation may have suggested many other potential candidate genes apart from *CRBP1*. Therefore, future rigorous investigation of these genes are warranted to elucidate radio-sensitive predictors in combination with *CRBP1*, which would be beneficial for selection of the most appropriate therapy for RC patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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