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Impact of p53 mutations on the synergistic effects of metformin and radiation on colorectal cancer cells

Kyoko Sakamoto, Koji Okabayashi, Yujin Kato, Kaoru Hattori, Satoru Morita, Kohei Shigeta, Yuko Kitagawa

Keio University School of Medicine, Tokyo, Japan

Abstract

Background Metformin has been reported to be effective as an adjunctive therapy in cancer treatment. In this study, the synergistic effects of metformin and radiotherapy were investigated in colorectal cancer cell lines and colorectal cancer organoids, as were the underlying mechanisms.

Methods This was an experimental study conducted *in vitro*. Cell proliferation was investigated *in vitro* after treatment of colorectal cancer cell lines with metformin and radiation. Western blotting was used to analyse treatment-induced changes in p53 expression. The combination index for metformin and irradiation was calculated. Organoids were generated via genomic analysis of colorectal cancer cells from surgical specimens from patients with colorectal cancer. Responses of colorectal cancer organoids with and without p53 mutations to metformin and radiation were compared. Organoids cultured from human colorectal cancer and cell lines were used.

Results In experiments using the colorectal cancer cell lines HCT116 and Lovo, which are p53 wild type cells, the combination treatment of metformin and irradiation appeared to have a synergistic effect. In HCT116 and Lovo cells, metformin treatment increased TP53 expression. No synergistic effect of metformin treatment and irradiation was demonstrated in WiDr and SW620 cells, which contain p53 mutations. Measurements of cell proliferation rates in colorectal cancer organoids also confirmed a similar trend in p53 wild-type organoids and those with p53 mutations.

Conclusion Mutations in the p53 gene in colorectal cancer cells may influence the synergistic effect of metformin and radiotherapy.

Keywords Colorectal cancer, metformin, radiation, organoid, p53

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Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm worldwide and the fourth leading cause of cancer-related death [1,2]. Among individuals with this disease, the 5-year survival rate for rectal cancer is less than 70%, while mortality rates among those under 50 years of age are reported to be increasing [3]. Multidisciplinary treatment has been used in recent years to improve the prognosis of rectal cancer patients. Combination treatment with surgery, chemotherapy and radiotherapy has been shown to improve recurrence-free survival rates [3-5]. Locally advanced rectal cancer, in particular, is more difficult to treat by radical surgery because of its anatomy, but preoperative chemoradiotherapy has been shown to contribute to a lower local recurrence rate [6]. Research into improving the effectiveness of adjuvant therapy for rectal cancer is important for improving patient outcomes.

Neoadjuvant therapy for rectal cancer usually involves chemoradiotherapy. Chemotherapy is administered as a radiosensitiser in neoadjuvant chemoradiotherapy (NACRT) protocols. One of the most frequently used NACRT protocols for rectal cancer is the combination of capecitabine and radiotherapy, where capecitabine enhances the effects of radiotherapy by causing cell cycle arrest and other effects [7]. The clinical complete response rate with NACRT is reported to be 10-30% [8-10], but total neoadjuvant therapy has recently

Department of Surgery, Keio University School of Medicine, Tokyo, Japan

Conflict of Interest: None

Correspondence to: Koji Okabayashi, MD, 35 Shinano-machi Shinjuku-ku Tokyo, Japan, e-mail: okabayashikoji@gmail.com

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been reported to increase this rate [11]. Dose limits have been established for radiotherapy to prevent secondary cancer and damage to other organs; thus, its effects cannot be improved simply by increasing the dose. [12]. The development of more effective radiosensitisers is therefore an important challenge in the treatment of rectal cancer.

Metformin is known to have anticancer properties, and patients treated with metformin have been reported to have a reduced incidence of cancer [13]. Furthermore, metformin has been shown to be useful as an adjuvant therapy for many cancer types. In contrast, metformin combined with chemoradiotherapy has demonstrated poor synergistic effects in some patient groups [14]. For metformin to be used effectively in cancer treatment, it is important to select a highly effective patient group. It has been suggested that mutations in the p53 gene may affect radiotherapy sensitivity and the efficacy of metformin. p53 is a tumor suppressor gene that causes cell cycle arrest and induces apoptosis [15]. Irradiation causes double-strand breaks in DNA and increases the expression of DNA repair proteins, including p53. p53 is downstream of the adenosine 5'-monophosphate kinase (AMPK) pathway, which is activated by metformin, and its expression has been reported to be altered by metformin treatment [16]. p53 mutations in tumor cells may influence the effects of metformin and irradiation, but this hypothesis has not been sufficiently investigated.

In this study, the synergistic effects of metformin and irradiation were investigated in CRC cell lines and CRC organoids, with and without genetic mutations in p53, to determine the differences in their effects.

Materials and Methods

Cell lines and reagents

The human CRC cell lines HCT116 (American Type Culture Collection, human colon carcinoma, CCL-247), SW620 (American Type Culture Collection, human colon carcinoma, CCL-227), Lovo (American Type Culture Collection, human colon adenocarcinoma, CCL-229), and WiDr (JCRB Cell Bank, human rectal adenocarcinoma, JCRB0224) were used in these studies and were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) under standard culture conditions of a humidified atmosphere of 5% CO_2 and 37°C. All tissue culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metformin (Sumitomo Dainippon Pharma, Tokyo, Japan, 250 mg/kg body weight) was prepared by dilution in 1× phosphate-buffered saline (PBS).

Cell proliferation assay

Cell proliferation was assessed by an MTT assay [17]. Cells were seeded in 96-well plates at a density of 4×10^4 cells/well. After 24 h, the cells were treated with metformin (0.1-10 mM); 1 h later, they were irradiated (1-4 Gy) using an AB-160 system (AcroBio Corporation, Tokyo, Japan). After 7 days of incubation, 10 μL of the SP cell count reagent SF (Nacalai Tesque, Kyoto, Japan) was added to each well, and the cells were further incubated for 2 h. The number of viable cells was directly proportional to the production of formazan following solubilisation. The colour intensity was measured at 450 nm in a Sunrise R microtiter plate reader (Tecan, Mannedorf, Switzerland). All experiments were performed in triplicate.

Western blot analysis

The cells were treated with 10 mM metformin and 2 Gy of radiation, and Western blot analysis was initiated the day after treatment. The cells were lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Proteins were quantified via a protein assay (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA), after which 20 μg of protein was separated via SDS-PAGE and transferred to a 0.2 μm nitrocellulose membrane. The membranes were blocked in tris-buffered saline, 0.1% Tween 20, and 5% bovine serum albumin for 2 h before an overnight incubation with primary antibodies diluted at 1:1000 in TBS, 0.1% Tween 20, and 5% BSA. Antibodies against p53, ataxia telangiectasia mutated, mammalian target of rapamycin, phospho-AMPK and beta-actin genes were obtained from Cell Signaling Technology (Beverly, MA, USA). The membranes were then incubated for 1 h with an horseradish peroxidase-conjugated secondary antibody diluted at 1:2000–1:10,000 in TBS and 0.1% Tween 20. Immunoreactive proteins were detected via MultiImage ll (Alpha Innotech, San Leandro, CA) [18]. Western blot densitometry quantification was performed using ImageJ.

Human tissue and genomic data

This study was approved by the ethics committee of Keio University (approval number: 20180015, 20211086) and was conducted in accordance with the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001. All patients provided written informed consent.

Tumor tissue was collected from surgical specimens of CRC patients who provided consent to undergo comprehensive genomic testing. Details of the panel have been previously reported [19-21].

Generation of patient-derived organoids

Organoids were established as previously reported, and surgical specimens were washed vigorously with PBS and minced into 1 mm^3 fragments using surgical scissors. The fragments were digested with Liberase TH (Roche, Switzerland) at 37°C for 30 min, and undigested pellets were further treated with TrypLE Express (Thermo Fisher Scientific, MA, USA) at 37°C for 10 min. Prior to plating, the cells and cell clusters were

washed with PBS supplemented with 10% FBS to inactivate digestive enzymes. Isolated cells were embedded in droplets of Matrigel Growth Factor Reduced Basement Membrane Matrix (Corning, NY, USA) and overlaid with the previously described basal culture medium for human intestinal organoids [22,23]. A complete culture medium was prepared by supplementing the basal culture medium with the following niche factors: 50 ng/mL mouse recombinant epidermal growth factor (Thermo Fisher Scientific, MA,USA), 50 ng/mL human recombinant fibroblast growth factor-2 (Peprotech, NJ, USA), 100 ng/mL human recombinant insulin-like growth factor-1 (BioLegend, CA, USA), 100 ng/mL mouse recombinant noggin (Peprotech, NJ, USA), 1 mg/mL recombinant human R-spondin-1 (R&D, MN, USA), human recombinant 100 ng/mL fibroblast growth factor-10 (Peprotech, NJ, USA) and 500 nM A83-01 (Tocris, UK). The plated organoids were maintained in an incubator with 5% CO_2 and 20% O_2, and the medium was changed every 5-7 days. Clinical and genomic information about organoids is listed in the Supplementary Table 1.

Cell viability assay

To analyse the response of the organoids to each drug, we plated 500-1000 organoid cells on 96-well plates. After 24 h, the cells were treated with metformin (1-10 mM), and 1 h later, they were irradiated (2-4 Gy) with an AB-160 system (AcroBio Corporation, Tokyo, Japan). Cell viability was measured on day 7 using a CellTiter-Glo® 3D Cell Viability Assay (Promega, WI, USA).

Combination index calculation

Data obtained from the MTT and cell viability assays were used to analyse the synergistic effects of metformin and radiation. Combination index (CI) analysis is a method used to assess drug interactions in cancer chemotherapy [24]. CI assesses drug interactions using a dose-based approach, by taking the ratio of concentrations showing 50% growth inhibition when 2 drugs are used alone and in combination [25]. The absorbance for each control group was converted to the fraction affected (Fa), which is the percentage of cell growth inhibition, and CompuSyn software version 1.0 (Combosyn Inc., Paramus, NJ, USA) was used to obtain the CI [24]. The drug used in this combination experiment was the non-contrast ratio, and the CI was automatically calculated via non-contrast ratio analysis using the software. CIs, which are indices used to evaluate the effects of drug combinations, can be classified into the following 3 categories: CI<1.0 is synergistic, CI=1.0 is additive, and CI>1.0 is antagonistic [24-26].

Data analysis

All the data are expressed as means ± standard errors. Data analyses were performed via 1-way and 2-way ANOVA using R (version 4.3.0 Vienna, Austria; R Foundation for Statistical Computing, 2023). Differences were considered statistically significant at $P<0.05$, and markedly significant differences were considered at $P<0.01$.

Results

Differences in the inhibitory effects of metformin and irradiation on cell proliferation in different cell lines

An MTT assay was performed to investigate the inhibitory effects of metformin and irradiation on cell proliferation *in vitro*. The metformin dose and radiation dose were combined, and the absorbance was measured on day 7 post-treatment. A non-metformin-treated group for each dose of irradiation served as the control group (Fig. 1). HCT116 and Lovo cells contain wild type p53, whereas WiDr and SW620 cells contain mutant p53 [27].

The cell proliferation rate was significantly lower in HCT116 and WiDr cells treated with 5 and 10 mM metformin and all doses of radiation. No clear inhibitory effect of metformin on cell proliferation was observed at most doses of radiation in Lovo or SW620 cells. Significant inhibition of cell proliferation was observed with 10 mM metformin only when Lovo cells were exposed to a dose of 2 Gy radiation (0.20 vs. 0.15, $P=0.0067$).

Evaluation of the synergistic effect of metformin and irradiation in different cell lines according to the combination index

Treatment effects were assessed according to the Fa. The Fa indicates 1 minus the survival fraction. The Fa was high in HCT116 cells, which indicates a strong therapeutic effect of metformin and irradiation (Fig. 2). The CI was measured via CompuSyn®. When 10 mM metformin was added to each cell line, the CI was <1 for HCT116 (1 Gy 0.128, 2 Gy 0.177 and 4 Gy 0.290) and Lovo (1 Gy 0.542, 2 Gy 0.245 and 4 Gy 0.346) cells, which suggests a synergistic effect. In WiDr cells, the CI was <1 (1 Gy 1.239, 2 Gy 1.302 and 4 Gy 1.478), which suggests an antagonistic effect between irradiation and metformin administration. For SW620 cells, the CI was ≈1 (1 Gy 0.862, 2 Gy 1.002 and 4 Gy 1.053), which suggests an additive effect.

Changes in TP53 expression induced by metformin in each cell line

Each cell line was treated with metformin alone, irradiation alone, or metformin and irradiation, and the p53 expression levels of each cell line were compared with those of untreated cells (Fig. 3). In HCT116 and Lovo cells, metformin increased TP53 expression, whereas in WiDr and SW620 cells, metformin reduced TP53 expression. The cell lines in which

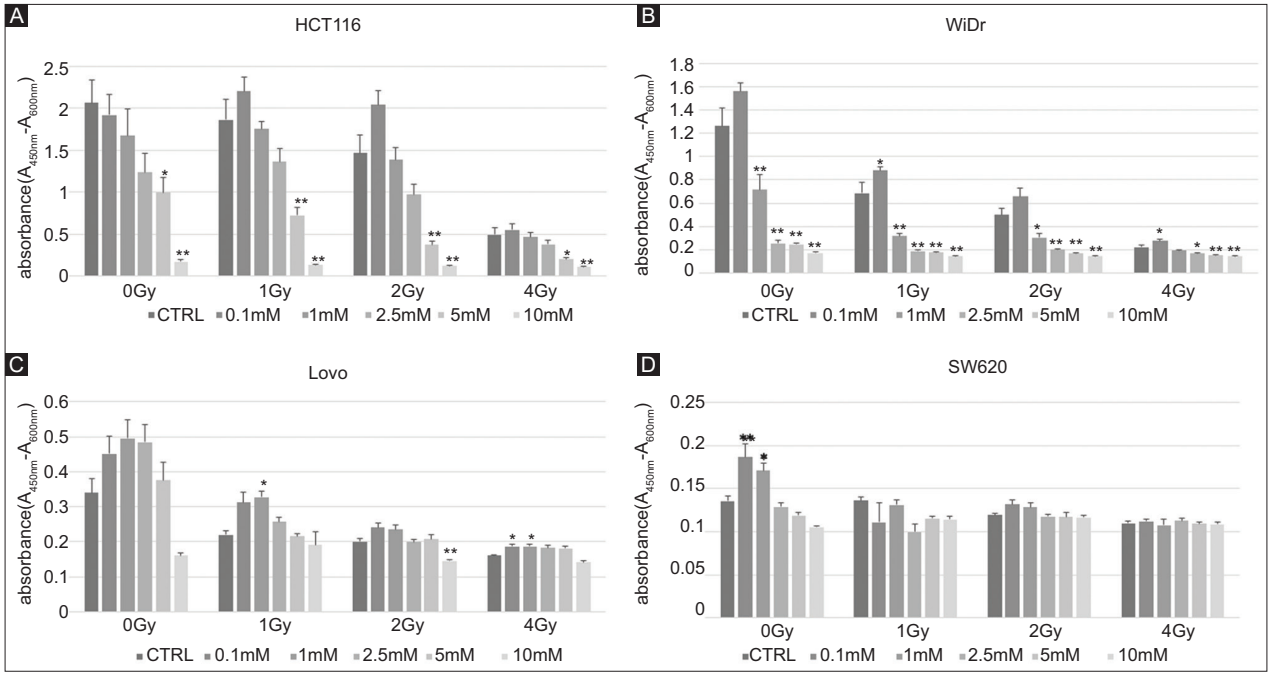

Figure 1 The combined effect of radiation and metformin on colorectal cancer cells. Evaluation of cell survival in (A) HCT116, (B) WiDr, (C) Lovo, and (D) SW620 cell lines by MTT assay. The assay was performed as 8 independent experiments in duplicate. The bars represent the mean values, and the error bars represent the SDs. Statistical analysis was performed via 1-way ANOVA with the Bonferroni *post hoc* correction
*P<0.05, **P<0.01
CTRL, control

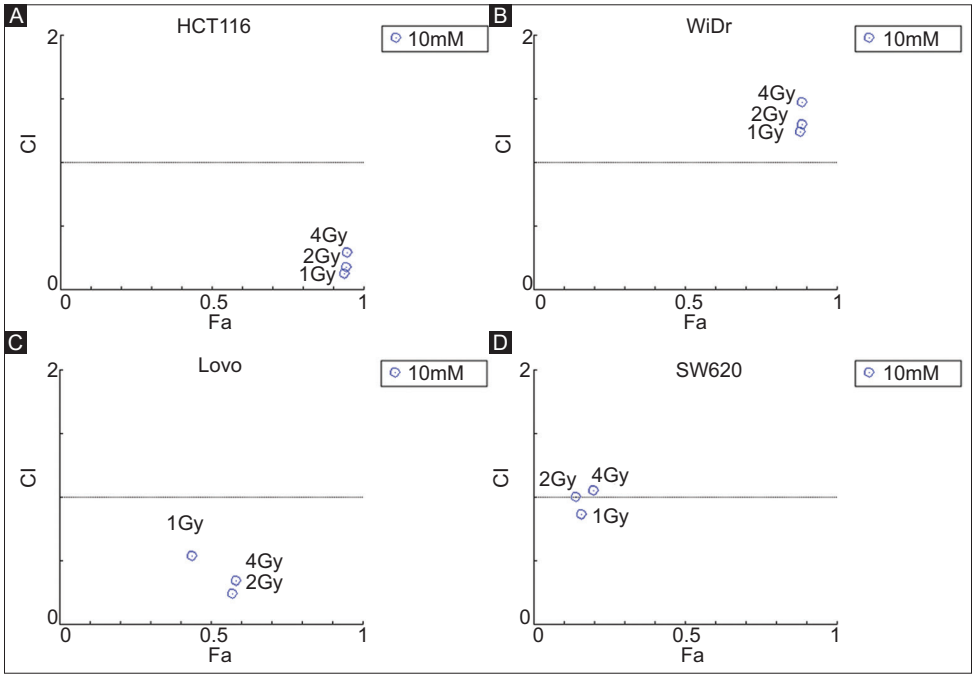

Figure 2 Fa-CI plot (Chou-Talalay plot) evaluating the synergistic effect between 10 mM metformin and radiation. Fa is the fraction affected, equal to 1 minus the survival fraction. The combination index (CI) value is calculated for the nonconstant ratio combinations. (A) HCT 116 and (C) Lovo have CIs <1 at any radiation dose, and thus metformin and radiation have synergistic effects. (B) WiDr has a CI>1, which indicates antagonism. (D) SW620 has a poor Fa, and its CI is nearly 1. This indicates only additive effects

metformin increased TP53 expression corresponded with the other cell lines for which CI calculations revealed a synergistic effect. Metformin increased phospho-AMPK expression and inhibited proliferation of HCT116 and WiDr cells, but

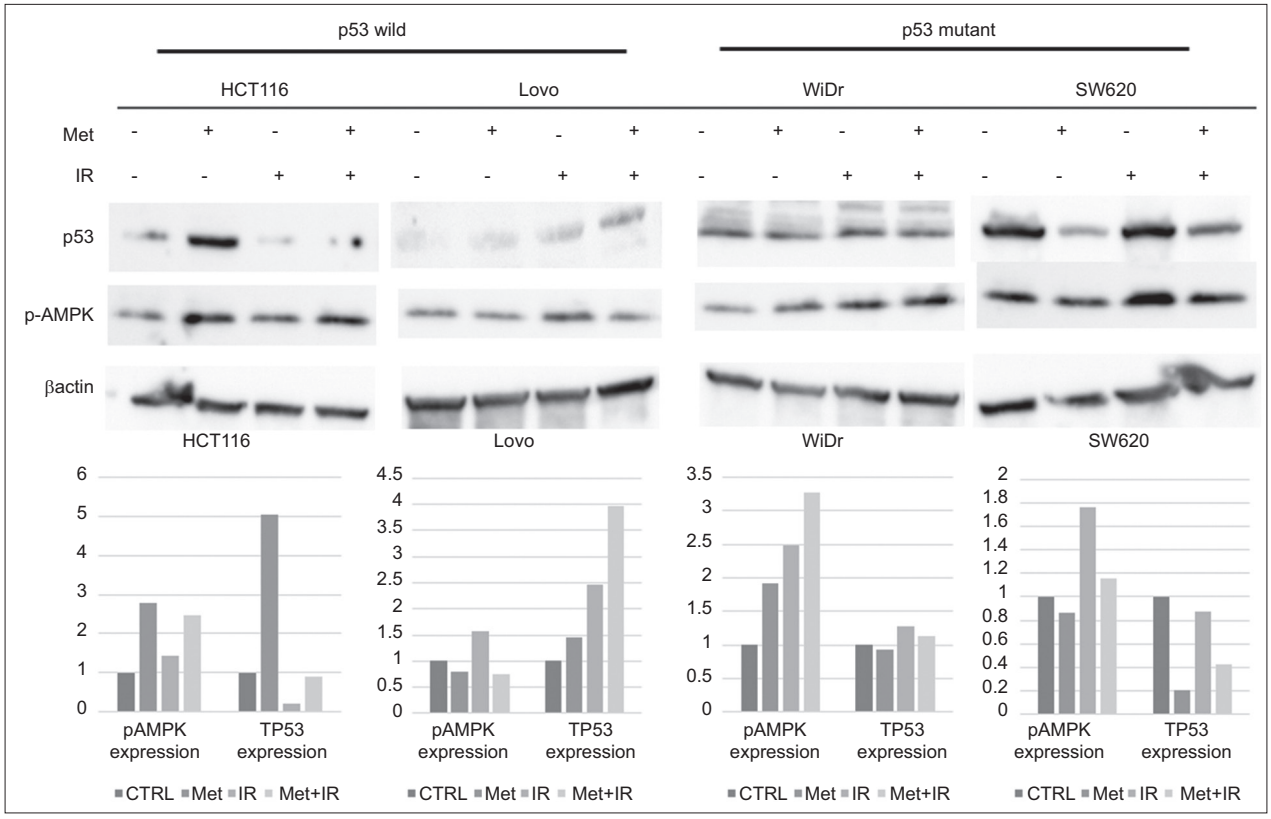

Figure 3 Western blot analysis demonstrating the expression patterns of p53, adenosine 5'-monophosphate kinase (AMPK), and phospho-adenosine 5'-monophosphate kinase (p-AMPK) following metformin treatment and radiation. Densitometric analysis of AMPK and TP53 in HCT116, Lovo, WiDr, and SW620 cells. Proteins were quantified and expressed as the ratio of the expression after each treatment relative to that of the control. The data are presented as the means ± standard errors of 2 independent experiments
*P<0.05 vs. the corresponding control, **P<0.01 vs. the corresponding control
CTRL, control

decreased phospho-AMPK expression and did not inhibit proliferation of Lovo and SW620 cells.

Therapeutic effects in p53 mutant organoids

Organoids were prepared from colon cancer cells collected from human colon cancer tissue. Each CRC cell line was subjected to a gene panel test to confirm the presence of p53 mutations. Colorectal cancer organoids were divided into 2 groups according to the presence or absence of mutations in the p53 gene, a tumor suppressor gene—p53 wild type (wt): T343, T370; p53 mutation type (mt): T329, T334. Each organoid was treated with metformin and was irradiated, and the cell proliferation rate was measured using a cell viability assay (Fig. 4). In p53 wt organoids, the inhibitory effect of metformin on cell proliferation was observed in the group treated with 10 mM metformin, with or without 2 Gy of radiation. In p53mt organoids, metformin was effective only when cells from the T343 organoids were irradiated with 2 Gy.

The CI was also measured to determine the rate of inhibition of cell proliferation in the organoids. In T334, the therapeutic effect of irradiation alone is limited, and the CI plot is provided for reference only (Supplementary Figures). In organoids with wt p53, a synergistic effect was confirmed when 2 Gy of irradiation was combined with 10 mM metformin, with a CI<1. No synergistic effect was observed for the T329 and T334 p53 mt organoids (CI≈1 for T329 and CI>1 for T334) (Fig. 5).

Discussion

The findings of this study support a synergistic effect of metformin and irradiation on p53, as observed in HCT116 and Lovo cells, which contain wild type p53, and whose TP53 expression increased with metformin treatment. Combined treatment also exerted synergistic effects on organoids generated from human CRC specimens, but only those with wild type p53. No synergistic effects were observed in CRC cell lines with mutant p53 or in p53 mt organoids. The combination therapy of metformin and irradiation in CRC cells suggested that p53 gene mutations and changes in TP53 expression may alter the therapeutic effect.

The efficacy of metformin as an adjuvant therapy for CRC has been reported in several studies. One clinical study, a meta-analysis comparing CRC patients who were treated with

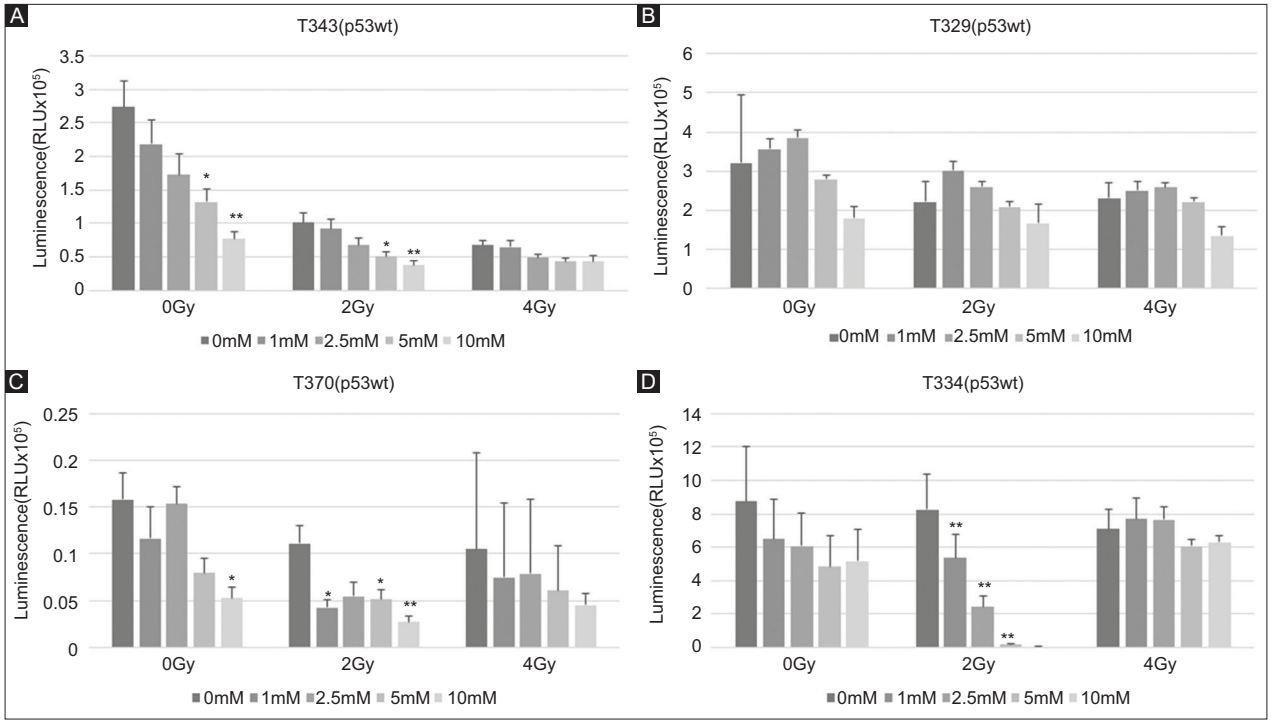

Figure 4 The combined effect of radiation and metformin on colorectal cancer organoids. Cell survival in organoids derived from human colorectal cancer tissue was determined using a cell viability assay. (A) (B) p53 wild-type, (C) (D) p53 mutation type organoids, as evaluated by sequencing. The assay was performed as 8 independent experiments in duplicate. The bars represent the mean values, and the error bars represent the standard deviations. Statistical analysis was performed via 1-way ANOVA with the Bonferroni *post hoc* correction
*P<0.05, **P<0.01
CTRL, control

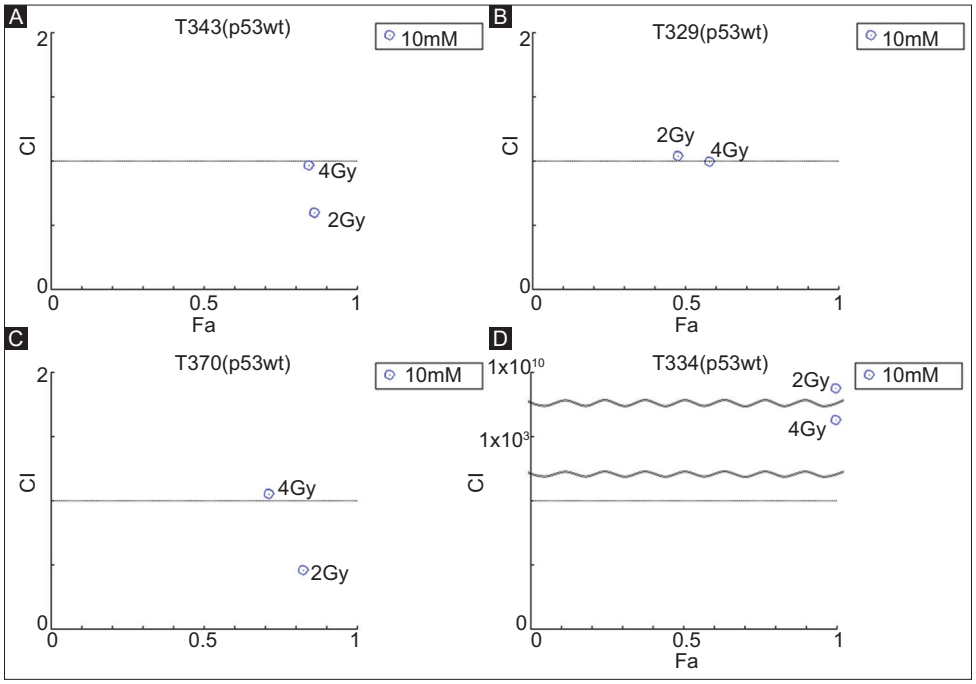

Figure 5 Fa-CI plot (Chou–Talalay plot) evaluating the synergistic effect of 10 mM metformin and radiation in colorectal cancer organoids. Fa is the fraction affected, equal to 1 minus the survival fraction. The combination index (CI) value is calculated for the nonconstant ratio combinations. (A, C) p53 wild-type organoids tend to have a CI<1 under 2Gy radiation, and thus metformin and radiation exert synergistic effects on these organoids. (B, D) p53 wild-type organoids have a CI=1 or CI>1, which suggests no synergistic effect

metformin perioperatively with those who were not treated with metformin, revealed that overall survival, recurrence-free survival and cancer-specific survival were superior in the metformin group [28]. We previously reported a meta-analysis showing that the effect of oral metformin in combination with NACRT is particularly strong in adenocarcinoma patients and in combination with 5-FU [14]. *In vivo* and *in vitro* experiments using CRC cell lines have also shown that media containing metformin, in combination with 5-FU, oxaliplatin and irradiation, has a significantly greater inhibitory effect on tumor growth than metformin-free media [29]. However, metformin functions as an mTOR inhibitor and has been shown to have antitumor effects when used alone. Whether the effects of metformin combined with other treatments for CRC are additive or synergistic is not clear.

Therefore, in the present study, the CI was used to examine whether the gains from metformin and irradiation were additive or synergistic. The CI has also been used in previous studies that have examined the combined effects of metformin and other drugs, and synergistic effects have been reported in colon cancer cells when metformin was combined with a dual PI3K and mTOR inhibitor [30]. This is the first report of a synergistic effect of metformin and irradiation in combination with the CI. Radiotherapy is an effective treatment option that can directly eliminate tumor cells without the invasiveness of surgery, but has the disadvantage of dose limits. In addition, irradiation is very effective for treating squamous cell carcinoma, but is less effective for treating adenocarcinoma [31]. The development of sensitisers that increase the efficiency of radiotherapy for rectal cancer is therefore an important challenge.

The mechanism by which metformin functions as a radiosensitiser has been described in several reports. Metformin has been shown to activate several antitumor signaling pathways through AMPK activation [32]. Metformin has also been shown to modify mitochondrial ROS metabolism in tumor cells, through the activation of AMPK and the suppression of mTOR, which may increase radiosensitivity [33]. Metformin is an old, inexpensive and widely used diabetes drug, which are important factors in its use as a radiosensitiser. The use of metformin as a radiosensitiser would become more feasible if it were possible to select patients who would derive greater benefits from metformin.

p53 is a tumor suppressor gene, and mutations in p53 are believed to affect the efficacy of radiotherapy. The ataxia telangiectasia mutated and ataxia telangiectasia and Rad3-related kinase genes are located upstream of p53 [34]. p53, which is phosphorylated by the protein products of these genes, has a wide range of activities as a transcription factor and induces the expression of many genes involved in DNA activity. Mutations in p53 may disrupt p53-mediated transactivation, cell cycle arrest and apoptotic responses [35]. HCT116 is a CRC cell line with wild-type p53, but studies have reported both complementary effects and no significant effects when irradiation is combined with metformin [36,37]. Reports have also been published on the effect of metformin as radiosensitiser on p53 knockout cells via small-interfering RNA and on the lack of that effect [36,38,39]. These studies did not use measures of synergistic effects, such as the CI, to determine the effect of irradiation alone or in combination with metformin. In addition, p53 can act as an adaptation-promoting protein in tumors [40]. Therefore, metformin has a wide array of effects that depend on the environment in which the individual cells are located, and on the presence of genetic mutations. Further studies are needed to clarify the interaction among p53, metformin and radiation, and would be of great clinical importance.

This study had several limitations. First, changes in the expression of many genes associated with p53 were not investigated. p53 is upstream and downstream of various gene expression cascades, and may indirectly affect other genes on which metformin essentially acts. Therefore, to investigate the isolated effects of p53 mutations, it will be necessary to use models such as p53 knockout mice. Second, organoids are not ideal human models. Compared with cell lines, organoids can partially recapitulate what occurs *in vivo* while maintaining diverse tumor environments, but organoids do not fully reflect the ecological environment. Third, metformin and radiation were administered directly to the cell lines and organoids in this study. *In vivo*, metformin is metabolised, and radiation exerts its anticancer effect by acting on the tissue surrounding the tumor. These differences in administration may have affected the behaviour and synergistic effects of metformin and irradiation.

In conclusion, the synergistic effect of metformin and irradiation in CRC may be influenced by p53 gene mutations and metformin treatment-induced changes in p53 expression.

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Summary Box

What is already known:

- Metformin and irradiation have an inhibitory effect on cancer cell growth
- Metformin and irradiation alter intracellular p53 expression
- The inhibitory effect of metformin on cancer cell growth depends on the cell line

What the new findings are:

- The findings of the combination index approach suggest that the simultaneous administration of metformin and irradiation has a synergistic effect
- The synergistic effects of metformin and irradiation appear to vary according to the p53 status of colorectal cancer cell lines
- In organoids derived from colorectal cancer specimens, the synergistic effect of metformin and irradiation was similar to the effect on colorectal cancer cell lines

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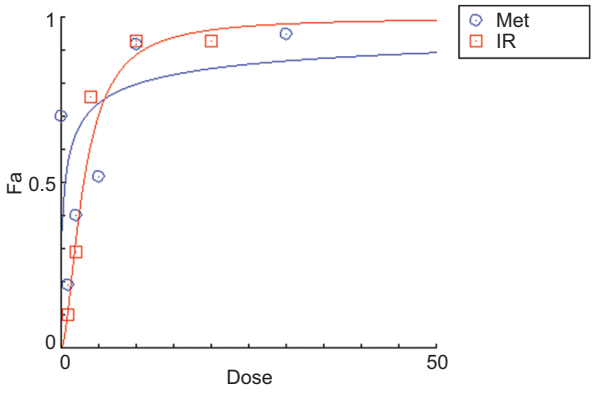
Supplementary material

HCT116



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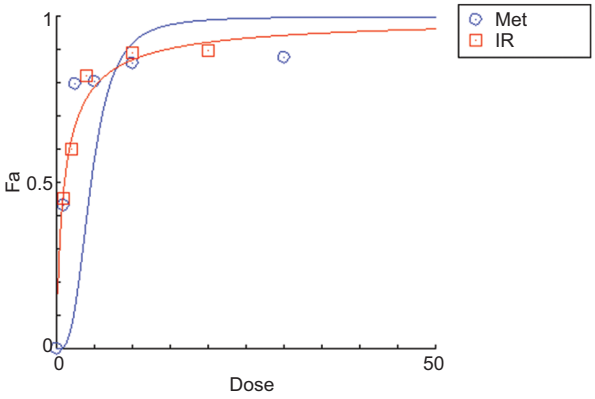

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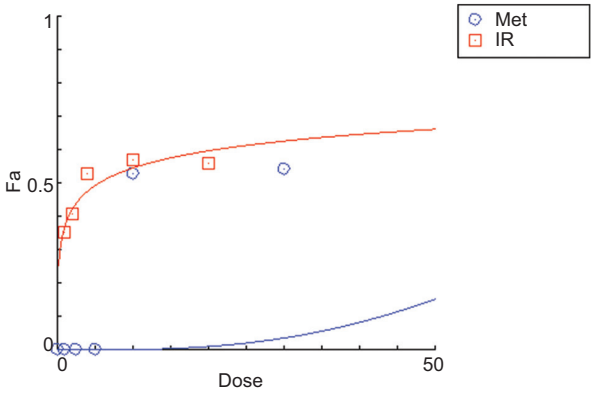
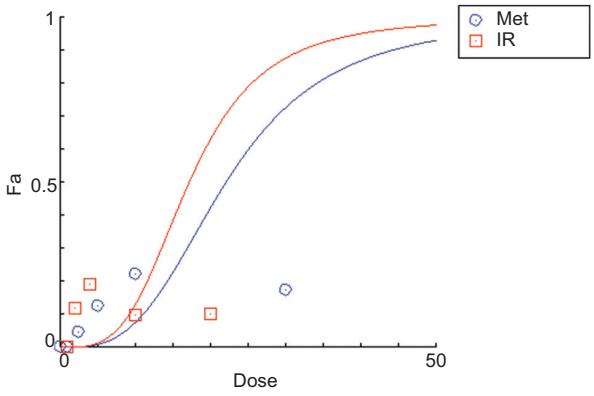
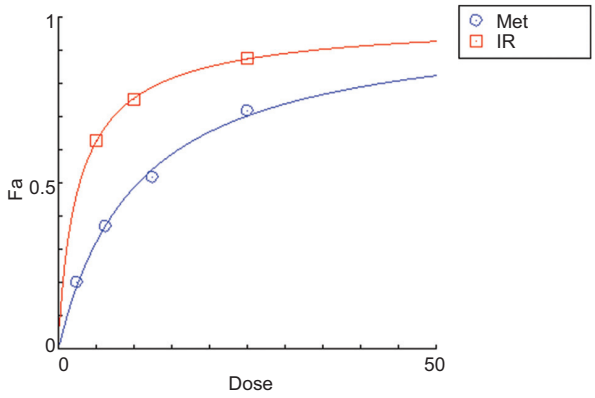


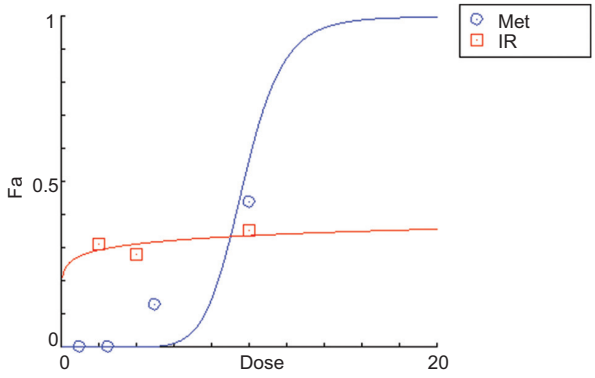
SW620

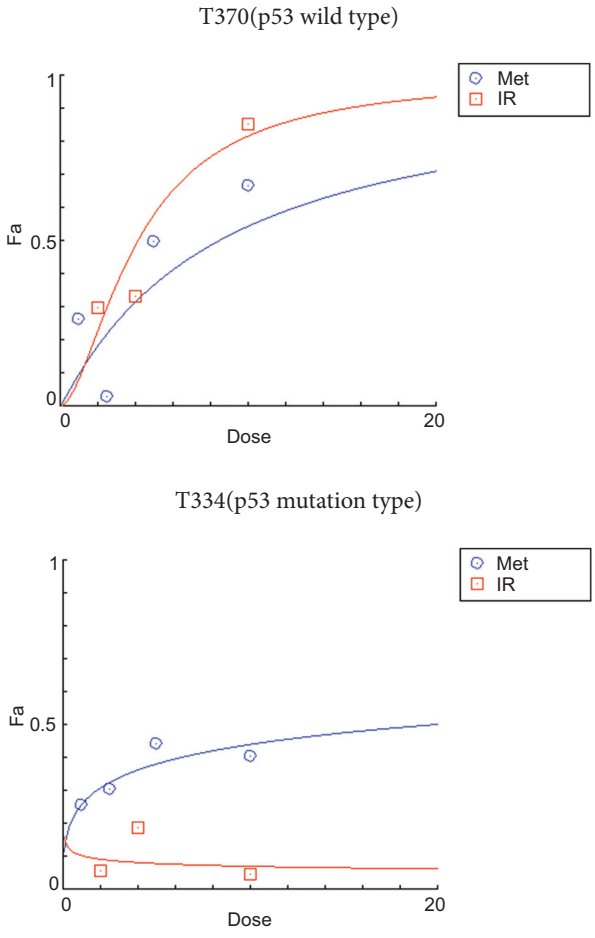


T343(p53 wild type)



T329(p53 mutation type)





Supplementary Table 1 Organoid information

	Age	Sex	location	Stage	Histological type	TMB	HRD	MSI	KRAS	p53
T343	42	Male	Sigmoid colon	IV	tub2	4	0.08	8.08	wild	wild
T329	60	Male	Rectum	II	tub2	10	0.24	8.67	wild	mutation
T370	57	Male	Ascending colon	I	tub1>tub2=muc	197	0.03	9.65	wild	wild
T334	80	Female	Rectum	III	tub2>muc	17	15.39	9.88	mutation	mutation

TMB, tumor mutational burden; HRD, homologous recombination deficiency; MSI, microsatellite instability