Introduction

Japanese Guidelines for the Treatment of Colorectal Cancer (2010) state that 5-fluorouracil (5FU)/leucovorin (LV) therapy, capecitabine, UFT/LV and FOLFOX4 and mFOLFOX6 are the standard treatments for postoperative adjuvant chemotherapy in stage III colon cancer in Japan [1]. The intravenous medications recommended in the guidelines cannot feasibly be used in the elderly and in patients for whom intensive therapy is not appropriate [2–4]. More effective, better tolerated and more convenient chemotherapy is required for these patients [5]. Moreover, a reported 84–89% of cancer patients would prefer oral to injected medications, given equivalent efficacy [6, 7].

Capecitabine is a novel oral fluorocytidine derivative discovered at the Nippon Roche Kamakura Laboratory (currently Chugai Pharmaceutical Co., Ltd. Kamakura Laboratory) that is designed to be converted to 5FU in steps to allow for selective delivery of high-dose 5FU to the target tumour while minimizing systemic exposure. After oral administration, capecitabine is absorbed unchanged from the gastrointestinal tract and metabolized in the liver to 5′-DFCR by carboxylesterase. The 5′-DFCR is subsequently converted to 5′-DFUR by cytidine deaminase, which is highly active primarily in the liver and tumour tissue. The 5′-DFUR is then selectively converted in the tumour tissue to 5FU by thymidine phosphorylase, which is highly active in tumour tissue [8, 9].

Based on the findings of capecitabine monotherapy in metastatic colon and rectal cancer, a phase III clinical trial (X-ACT trial) was conducted to compare capecitabine to bolus 5FU/LV therapy (Mayo Clinic regimen) as adjuvant chemotherapy in resected stage III colon cancer. Twelves et al. [10] reported that the capecitabine therapy in the study was at least equivalent to 5FU/LV therapy (Mayo Clinic regimen) in terms of the primary endpoint of 3-year disease-free survival (DFS) as well as overall survival (OS). In addition, capecitabine was associated with fewer gastrointestinal disturbances such as diarrhoea and stomatitis, indicating a superior safety profile [10]. Capecitabine was approved in the US and Europe in 2005 based on these results. Currently, the NCCN Guidelines include capecitabine monotherapy among the standard treatments for postoperative adjuvant chemotherapy in stage III colon cancer, along with 5FU/LV therapy and 5FU/LV + oxaliplatin therapy [11].

Occasionally, the treatment must be suspended due to the development of the characteristic adverse reaction of hand–foot syndrome (HFS). The treatment completion rate was 83.0% in the X-ACT trial, in which capecitabine was recognized as a standard postoperative adjuvant chemotherapy for colorectal cancer. Although capecitabine was approved in Japan based on the results of this trial, it has not yet been established to be a safe postoperative adjuvant therapy for colon cancer in Japanese patients. Accordingly, a feasibility study with an endpoint of the treatment completion rate of capecitabine in Japanese patients was planned.

Patients and methods

Inclusion criteria

Patients who met all of the following criteria were eligible for the study regardless of sex. (1) Histologically-confirmed colorectal cancer (adenocarcinoma). (2) Histological stage III [12] colon cancer or rectosigmoid cancer. (3) Curative colorectal cancer resection with D2 or more lymph node dissection. (4) Surgical procedure classified as histological curability A (cur A) [12] was performed. (5) Age 20–80 years old. (6) Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1. (7) No prior chemotherapy or radiotherapy for target disease. (8) Oral intake is possible. (9) Preservation of primary organ function [white blood cell (WBC) count $\geq$3,000/mm^3 and $<$12,000/mm^3, neutrophil count $\geq$1,500/mm^3, haemoglobin $\geq$9.0 g/dL, platelet count $\geq$100,000/mm^3, serum creatinine $\leq$1.5 times upper laboratory reference, serum total bilirubin $\leq$1.5 times upper laboratory reference, aspartate aminotransferase (AST), alanine aminotransferase (ALT) $\leq$2.5 times upper laboratory reference, alkaline phosphatase (ALP) $\leq$2.5 times upper laboratory reference]. (10) Able to start protocol treatment within 8 weeks of surgical procedure. (11) After basic screening, informed consent to participate in the study was obtained from patients. This study was approved by the Ethics Committees of the participating institutions and registered in the UMIN clinical trials registry (UMIN000001444).

Exclusion criteria

Patients who met any of the following criteria were excluded from participation in the study. (1) Pregnant or lactating, or planning to become pregnant. (2) History of hypersensitivity or severe adverse reaction to fluoropyrimidines. (3) Past organ transplant. (4) Serious concurrent disease [including interstitial pneumonia, pulmonary fibrosis, intestinal paralysis, ileus, poorly controlled diabetes, liver cirrhosis or hepatitis (type B or C), poorly controlled hypertension, history of myocardial infarction or unstable angina within past 6 months]. (5) Active multiple primary cancer (disease-free less than 5 years). (6) Concurrent infectious disease. (7) Judged to be unsuitable for participation in the clinical study by the investigator for any other reason.

Staging criteria

Japanese Classification of Colorectal Carcinoma, 7th edition (Second English edition) [12] was used for staging.

Treatment and assessment

Patients were enrolled and started on the protocol treatment within 8 weeks postoperatively. The protocol treatment consisted of eight 3-week cycles of 2,500 mg/m^2 of capecitabine per day in two divided doses for 14 days, followed by a 7-day rest period. To assess the protocol treatment, baseline tumour markers, peripheral blood counts and blood chemistry values were measured within 14 days of the start of administration.

General findings, peripheral blood counts, blood chemistry values and clinical findings (subjective symptoms and objective signs) were generally recorded at each visit during treatment. To assess HFS, photos of both the palms and the soles of the feet of patients were taken at baseline, at the onset of each grade of HFS and at recovery, with consent of the patient.

Protocol discontinuation criteria

Patients who met any of the following criteria were discontinued from the protocol treatment. (1) Onset of adverse event meeting the criteria for discontinuation of capecitabine in "dose modification". (2) Did not recover sufficiently to meet the start criteria for the next cycle of capecitabine even though 3 weeks had passed from the scheduled date of the start of the next cycle due to onset of adverse event in previous cycle. (3) 3 weeks had passed from the scheduled start date of the next cycle for reasons other than an adverse event (such as patient circumstances) (discontinuation of protocol treatment not required if treatment could be resumed without compromising efficacy). (4) Attending physician determines discontinuation of protocol treatment is necessary for reasons related to adverse event that does not meet the discontinuation criteria. (5) Confirmed recurrence. (6) Death. (7) Patient requests discontinuation from the protocol treatment for adverse event-related reason (relation to an adverse event cannot be ruled out). (8) Patient requests discontinuation from the protocol treatment for reason unrelated to adverse event [relation to an adverse event can be ruled out (e.g., relocation of patient or patient's family)]. (9) Patient found to have been ineligible for participation in the study after enrolment. (10) Pregnancy. (11) Confirmed secondary cancer or multiple cancers. (12) Investigator or primary or attending physician judges continuation of treatment to be inappropriate for any other reason.

Statistical analysis

The objective of the current study was to assess the feasibility of postoperative adjuvant chemotherapy with capecitabine for patients with stage III colon cancer. The primary endpoint was the treatment completion rate. The secondary endpoints were safety profile (rate and severity of adverse events), cumulative incidence of HFS and hepatic dysfunction (secondary analyses), 3- and 5-year DFS and OS.

Protocol completion was defined as the completion of eight 3-week cycles of 14 days of capecitabine followed by a 7-day rest period according to protocol in patients who were enrolled and started on treatment within 8 weeks postoperatively. If the eighth cycle was suspended, a ninth cycle was planned for patients meeting the treatment resumption criteria. These patients were considered to have completed eight cycles. Completion was judged by a central assessment committee in cases of protocol deviations or violations.

The objective of the current study was to find the estimated treatment completion rate in the patient population meeting the enrolment criteria for treatment with capecitabine as postoperative adjuvant therapy for colon cancer. The treatment completion rate was 83% in the X-ACT trial conducted outside of Japan using the same regimen [10]. Although the exact number of completing patients is unknown, the estimated 95% confidence interval (CI) was 80–86%. The study was estimated to require a sample size of at least 87 patients to ensure that the 95% confidence limit of the estimated treatment completion rate was within 10% of either side of the estimated value (if 70 of the 87 patients completed treatment, the estimated observation rate, or the estimated treatment completion rate, would be 80.5% with a 95% CI of 70.6–88.2%, resulting in a 9.9% difference between the estimated value and the lower confidence limit). A target enrolment size of 92 was selected, to account for patient attrition of approximately 5% due to ineligibility or other reasons.

Results

Patient baseline characteristics

From August 2008 to August 2009, 97 patients were enrolled at 42 centres belonging to the Kyushu Study Group of Clinical Cancer (KSCC). The per protocol set (PPS) excluded six of these patients due to incorrect administration. The patients had a median age of 65 years (range, 32–80 years). Males accounted for 60 of the patients, and females for 37. Surgical history included D2 lymph node dissection in 25 patients and D3 lymph node

Table 1 Patient characteristics

Characteristics	No. of patients
Age, years	
Median (range)	65 (32–80)
Gender	
Male/female	60/37
PS	
0/1	93/4
Lymph node dissection	
D0/D1/D2/D3	0/0/25/72
Primary tumor	
C/A[a]/T/D/S/RS	8/11/9/15/32/22
Stage	
IIIa/IIIb	74/23
Invasion	
SM/MP/SS/A[b]/SE/SI/AI	6/6/58/3/21/2/1
Nodal status	
N1/N2/N3	74/19/4
Regional lymph nodes	
ly0/ly1/ly2/ly3	21/47/21/8
Venous invasion	
v0/v1/v2/v3	27/45/18/7
Histological classification	
pap/tub1/tub2/por/muc/sig	2/31/59/3/2/0

C cecum, A^a ascending colon, *T* transverse colon, *D* descending colon, *S* sigmoid colon, *RS* rectosigmoid, *SM* carcinoma is limited to within the mucosa and submucosa, *MP* carcinoma is limited to within the mucosa submucosa, and proper muscle layer, *SS* carcinoma extends from the mucosa beyond the proper muscle but is not exposed on the serosal surface, A^b carcinoma extends beyond the proper muscle, *SE* carcinoma is exposed on the serosal surface, *SI* carcinoma definitely infiltrates other organs, *AI* carcinoma definitely infiltrates other organs, *pap* papillary adenocarcinoma, *tub1* well differentiated type (tubular adenocarcinoma), *tub2* moderately differentiated type (tubular adenocarcinoma), *por* poorly differentiated adenocarcinoma, *muc* mucinous adenocarcinoma, *sig* signet-ring cell carcinoma

dissection in 72 patients. Nodal status was N1 in 74 patients, N2 in 19 patients and N3 in four patients (Table 1).

Treatment completion rate

The treatment completion rate in the full analysis set (FAS), which was the primary endpoint, was 66.0% (64/97). The treatment completion rate in the PPS was 70.3% (64/91). Treatment was discontinued in 33 patients. Treatment was discontinued for an adverse event in 24 of these patients. The most common adverse event resulting in treatment discontinuation was HFS (seven patients), followed by haematological toxicities and liver dysfunction (five patients each). The reasons for treatment discontinuation in the remaining nine patients were incorrect administration in six patients and recurrence in three patients (Table 2).

Table 2 Reasons for protocol discontinuation

Reasons	*N*
Adverse events	
Hand–foot syndrome	7
Haematological toxicities	5
Liver dysfunction	5
Fatigue	4
Diarrhoea	2
Rash	1
Extrapyramidal symptom	1
Skin ulcer	1
Ileus	1
Nausea/vomiting	1
Others	
Protocol violations/administrative reasons	6
Protocol violation/regimen changed	1
Relapse	3

Table 3 Most common treatment-related adverse events

Events	All grades (%)	Grade 3/4 (%)
Diarrhoea	23 (23.7)	2 (2.1)
Stomatitis	30 (30.9)	0 (0)
Hand–foot syndrome	64 (66.0)	22 (22.7)
Nausea	26 (26.8)	1 (1.0)
Vomiting	7 (7.2)	1 (1.0)
Neutropenia	56 (57.7)	7 (7.2)
Hyperbilirubinaemia	44 (45.4)	0 (0)
ALT	34 (35.1)	0 (0)
AST	33 (34.0)	0 (0)

ALT alanine aminotransferase, *AST* aspartate aminotransferase

Safety

The most common treatment-related adverse events were diarrhoea 23 (23.7%), stomatitis 30 (30.9%), HFS 64 (66.0%), neutropenia 56 (57.7%), and nausea/vomiting 33 (34.0%) (Table 3). The major grade 3/4 adverse events were HFS 22 (22.7%), neutropenia 7 (7.2%) and diarrhoea 2 (2.1%). Extrapyramidal symptoms and skin ulcer also occurred in one patient each.

Dose modification

Three dose levels were used for dose modification (levels 0, 1, 2). Patients were discontinued if adverse events could not be controlled at −2 levels. An analysis of dose

modification at the end of cycle 8 showed that 40.2% (39) of the patients did not require dose reduction, 34% (33) of the patients were discontinued, 7.2% (7) of the patients had dose reductions of −2 levels and 18.6% (18) of the patients had dose reductions of −1 level.

The most common adverse event requiring dose modification was HFS. By the end of cycle 4, dose modification was required by 78.4% (40/51) of the patients with grade 1 HFS, 60% (3/5) of the patients with grade 2 HFS and 87.5% (7/8) of the patients with grade 3 HFS.

Discussion

To allow for the use in Japan of the dosing regimen based on non-Japanese evidence (2,500 mg/m^2 for 2 weeks followed by 1-week rest period), a late Japanese phase II clinical trial (JO15951) was conducted in advanced metastatic colon and rectal cancer using this established dosing regimen [13]. Results were obtained equivalent to those obtained in the non-Japanese phase III clinical trials in advanced colorectal cancer (SO14695, SO14796) [14, 15]. In addition, no safety issues of concern were identified. Capecitabine was therefore approved as a postoperative adjuvant therapy for colon cancer at this dosage in Japan in December 2007 based on the results of the non-Japanese clinical trial of postoperative adjuvant chemotherapy in stage III colon cancer (X-ACT trial), a randomized controlled trial in which capecitabine was recognized as a standard treatment in colon cancer. Comparison of the safety data from the non-Japanese phase III clinical trials in patients with advanced metastatic colorectal cancer (SO14695, SO14796) and the Japanese phase II clinical trial (JO15951) showed that while the adverse reaction profile of capecitabine was similar in the Japanese and non-Japanese trials, the frequency of some adverse reactions varied. It was therefore extremely important to evaluate the safety of the dosing regimen of 2,500 mg/m^2/day of capecitabine for 2 weeks followed by a 1-week rest period for postsurgical adjuvant therapy in Japanese patients with stage III resected colon cancer.

The current feasibility study was conducted due to the lack of data on postoperative adjuvant chemotherapy with capecitabine in Japanese patients. The treatment completion rate was 66.0% (64/97) in the FAS and 70.3% (64/91) in the PPS. These rates were lower than the 83.0% rate observed in the X-ACT trial. The discrepancy is likely due to differences in the discontinuation criteria between the X-ACT trial and the current study. The discontinuation criteria of the present study were constructed based on those of a late Japanese phase II clinical trial (JO15951) conducted in advanced metastatic colorectal cancer. Consequently the following criteria were added to those of the

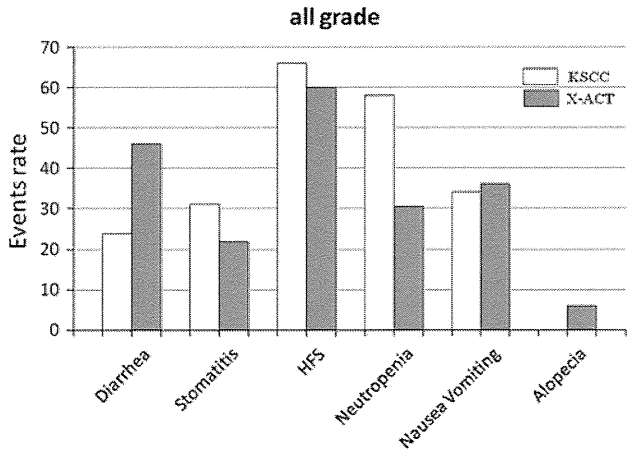

Fig. 1 Main toxicities of KSCC0803 compared to X-ACT trial

X-ACT trial: capecitabine administration was not restarted within 3 weeks; pregnancy; and secondary or multiple cancers. When the discontinuation criteria of the X-ACT trial were used, the treatment completion rate was 75.3% (73/97) in the FAS and 80.2% (73/91) in the PPS. These findings suggest that capecitabine is tolerable in Japanese patients.

A comparison of the most common adverse events in the X-ACT trial and the present study showed that diarrhoea occurred with a lower incidence and neutropenia occurred with a higher incidence in the current study than the X-ACT trial. These patterns are similar to those previously reported for colorectal cancer chemotherapy and are not therefore solely characteristic of capecitabine (Fig. 1) [16].

Most of the cases of the dose-limiting toxicity HFS occurred before the end of cycle 4, suggesting that active monitoring is required during the first half of treatment. The analysis of DFS by dose intensity (DI) in the X-ACT trial showed that the DFS rate was comparable for DI of >90% and DI of 60–90% [17]. This finding emphasizes the importance of active individual dose reduction to ensure proper management of continued treatment.

Our results confirm those of previous global phase III studies and show that capecitabine is well tolerated in both global and Japanese-only populations, with similar high completion rates in both. The X-ACT trial indicated that capecitabine DI made no difference to DFS. There was no difference between DI >90% and a DI ≥60 and ≤90%. These findings suggest that it might be important to complete treatment by controlling adverse effects through effective dose modification. Survival follow-up of this study is now ongoing.

Acknowledgments We are indebted to the physicians and all other co-medical staff who contributed to this study. We also thank Ms. Taniguchi, Ms. Kozuru, and Ms. Kasamoto at the Clinical Research Support Center Kyushu (CReS Kyushu) for their excellent data management and secretarial assistance.

Conflict of interest Yoshihiko Maehara has received research funding from Chugai Pharmaceutical. All other authors declare no conflicts of interest.

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17. F. Hoffmann-La Roche, data on file

DNA Repair 11 (2012) 247–258

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

ATR–Chk1 signaling pathway and homologous recombinational repair protect cells from 5-fluorouracil cytotoxicity

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ARTICLE INFO

Article history:
Received 14 June 2011
Received in revised form
20 November 2011
Accepted 25 November 2011
Available online 20 December 2011

Keywords:
5-Fluorouracil
Double-strand breaks
Homologous recombinational repair
Chk1
Rad9
Rad17

ABSTRACT

5-Fluorouracil (5-FU) has long been a mainstay antimetabolite chemotherapeutic drug for the treatment of major solid tumors, particularly colorectal cancer. 5-FU is processed intracellularly to yield active metabolites that compromise RNA and DNA metabolism. However, the mechanisms responsible for its cytotoxicity are not fully understood. From the phenotypic analysis of mutant chicken B lymphoma DT40 cells, we found that homologous recombinational repair (HRR), involving Rad54 and BRCA2, and the ATR–Chk1 signaling pathway, involving Rad9 and Rad17, significantly contribute to 5-FU tolerance. 5-FU induced γH2AX nuclear foci, which were colocalized with the key HRR factor Rad51, but not with DNA double-strand breaks (DSBs), in a dose-dependent manner as cells accumulated in the S phase. Inhibition of Chk1 kinase by UCN-01 increased 5-FU-induced γH2AX and enhanced 5-FU cytotoxicity not only in wild-type cells but also in *Rad54*- or *BRCA2*-deficient cells, suggesting that HRR and Chk1 kinase have non-overlapping roles in 5-FU tolerance. 5-FU-induced Chk1 phosphorylation was significantly impaired in *Rad9*- or *Rad17*-deficient cells, and severe γH2AX nuclear foci and DSBs were formed, which was followed by apoptosis. Finally, inhibition of Chk1 kinase by UCN-01 increased 5-FU-induced γH2AX nuclear foci and enhanced 5-FU cytotoxicity in *Rad9*- or *Rad17*-deficient cells. These results suggest that Rad9- and Rad17-independent activation of the ATR–Chk1 signaling pathway also significantly contributes to 5-FU tolerance.

Abbreviations: 5-FU, 5-fluorouracil; FUTP, fluorouridine triphosphate; FdUTP, fluorodeoxyuridine triphosphate; FdUMP, fluorodeoxyuridine monophosphate; TS, thymidylate synthase; BER, base excision repair; MMR, mismatch repair; DSB, double-strand break; HRR, homologous recombinational repair; ATR, ataxia telangiectasia-mutated and Rad3-related kinase; ATRIP, ATR interacting protein; FA, Fanconi anemia; RPA, replication protein A; ssDNA, single-strand DNA; 9-1-1, Rad9-Hus1-Rad1; RFC, replication factor C; O^6-meG, O^6-methylguanine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; BrdU, bromodeoxyuridine; PI, propidium iodide.

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1568-7864/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2011.11.005

1. Introduction

5-Fluorouracil (5-FU) is a fluoropyrimidine-type compound with marked anti-tumor effects [1]. For many years, 5-FU has been an important chemotherapeutic drug used in first-line treatment of a range of human cancers, particularly colorectal carcinoma [2]. Intracellularly, 5-FU is converted to several active metabolites, including fluorouridine triphosphate (FUTP), fluorodeoxyuridine triphosphate (FdUTP) and fluorodeoxyuridine monophosphate (FdUMP). These active metabolites compromise global RNA metabolism by incorporation of FUTP into RNA and DNA metabolism by FdUMP-mediated inhibition of thymidylate synthase (TS) and incorporation of FdUTP into DNA [2]. Inhibition of TS is achieved by the formation of a ternary covalent complex consisting of TS-FdUMP-5,10-methylenetetrahydrofolate. Once this complex is formed, cells are unable to synthesize dTMP from dUMP, and the cellular dUTP level increases at the expense of dTTP. The resulting dUTP/dTTP imbalance causes massive misincorporation of dUTP or FdUTP, particularly during DNA replication.

Although DNA damage is considered to be one of the main triggers of the tumor cell killing effects of 5-FU [3,4], it is not fully understood how misincorporated dUTP or FdUTP are processed and contribute to cytotoxicity. Misincorporated FdUTP or dUTP are recognized, excised from DNA, and undergo base excision repair (BER) or mismatch repair (MMR) [5]. DNA strand breaks are generated as byproducts of the repair processes. Homologous recombinational repair (HRR) has been proposed to contribute to the repair of 5-FU-induced DNA damage, including double-strand breaks (DSBs) [3], but no direct evidence for this process has been obtained in vertebrate cells. In addition, 5-FU activates Chk1 kinase, the main mediator of the activation of cell cycle checkpoints and DNA repair in response to genotoxic stress [6], which is achieved via phosphorylation at Ser317 and Ser345 by ataxia telangiectasia-mutated and Rad3-related kinase (ATR) [7]. The critical role of Chk1 in 5-FU resistance has been reported in human cancer cell lines [8], the chicken DT40 cell line [9] and a mouse xenograft model [10]. Importantly, Chk1 elimination by siRNA in HeLa cells abolishes the S-phase checkpoint induced by 5-FU. This allows DNA synthesis to continue, leading to excessive accumulation of DNA DSBs, and ultimately potentiates the efficacy of 5-FU [8].

ATR is mainly activated in the replication protein A (RPA)-coated single-strand DNA (ssDNA) region by anchoring ATR interacting protein (ATRIP) [11]. Rad9 and Rad17 are essential for the activation of ATR and Chk1 induced by RPA-coated ssDNA [7]. Rad9 is a component of the Rad9-Hus1-Rad1 (9-1-1) complex, a PCNA-like clamp that is loaded at the boundary of dsDNA and ssDNA [12]. The 9-1-1 complex is required for the recruitment of TopBP1 [13], which activates the ATR–ATRIP complex [14]. Rad17 forms a complex with replication factor C (RFC) 2-5 and loads the 9-1-1 clamp to sites of DNA damage [15]. ATR is also activated by direct binding to the MutSα complex, which binds to O^6-methylguanine (O^6-meG):dTTP adducts generated by S_N1-type alkylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [16–18]. MMR factors, but not RPA or Rad17, are required for ATR-mediated SMC1 phosphorylation by MNNG [18]. ATR and MMR factors, but not RPA, are recruited to O^6-meG:dTTP adducts *in vitro* [16,17]. A MMR-mediated cell cycle checkpoint response to fluorodeoxyuridine has also been reported [19], but the contribution of this response to cellular tolerance to 5-FU is not clear.

To elucidate the mechanisms responsible for the activation of the DNA damage response by 5-FU, we determined 5-FU sensitivity and cellular responses in chicken B lymphoma DT40 cells and in gene knockout strains as model systems. We found that mutants of HRR factors (*Rad54* and *BRCA2*) and of factors involved in the ATR–Chk1 signaling pathway (*Rad9* and *Rad17*) exhibited significantly increased sensitivity to 5-FU. Consistently, 5-FU induced nuclear foci of phosphorylated histone H2AX (γH2AX), which colocalized with HRR factor Rad51, but not with DNA DSBs. From the phenotypic analysis of these mutant cells and cells whose Chk1 activity was inhibited by UCN-01, we revealed the critical role of the ATR–Chk1 signaling pathway and HRR for protecting cells from 5-FU cytotoxicity.

2. Materials and methods

2.1. Cell culture and chemicals

Chicken DT40 and their mutant cells (*rad9*, *rad17* [20], *rad54* [21], *ku70* [22], *brca2ΔCTD* [23] and *fancd2* [24]) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, penicillin and streptomycin in a 5% CO_2 incubator at 39.5 °C. The *rad54* and *ku70* strains were kindly provided by Dr. Shunichi Takeda. 5-FU and UCN-01 were purchased from Sigma (St. Louis, MO).

2.2. Western blotting and antibodies

We used antibodies against Chk1 (G-4, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Chk1 at serine 345 (pS345; 133D3, Cell Signaling, Boston, MA), β-actin (Sigma), TS (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), and chicken FancD2 (kindly provided by Dr. Kenshi Komatsu, Kyoto University, Japan). Pellets of treated DT40 and mutant cells were dissolved in sample buffer. The protein concentration was measured by the Lowry method using RC DC protein assay kit (BioRad, Hercules, CA) and 10 μg of total cell extract was subjected to SDS-PAGE and western blotting. Chemiluminescent signals were captured by ImageQuant™ LAS-4000 (GE Healthcare UK Ltd., Buckinghamshire, UK) and signal intensity was quantified by Image-J software (NIH).

2.3. Clonogenic survival assay

The clonogenic survival assay for 5-FU was carried out as previously described [9]. Briefly, cells were cultured with media containing the indicated concentration of 5-FU for 16 h and then washed twice with fresh media. Trypan blue-negative cells were counted by a hemocytometer and an appropriate number of cells was inoculated in medium containing 1.4% methylcellulose. After 1–2 weeks of culture in a 5% CO_2 incubator at 39.5 °C, the emerging colonies were counted. Experiments were done in triplicate.

2.4. Fluorescence immunostaining

After exposure to 5-FU, cytospin slides were fixed with 4% paraformaldehyde and immunostained with antibodies against chicken FancD2, human Rad51 (H-300, Santa Cruz Biotech) and γH2AX (phospho-Ser139 H2AX (JBW301), Millipore, Billerica, MA). To detect γH2AX foci, 4% paraformaldehyde-fixed cells were treated with 70% ethanol before immunostaining, as previously described [23]. Cells were stained with an Alexa555-conjugated secondary antibody (Invitrogen, Carlsbad, CA) for single staining and the nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI). Nuclei containing five or more foci were counted as foci-positive and more than 500 nuclei were examined in each condition. Double staining was done with Alexa488- and Alexa594-conjugated secondary antibodies. All images were captured using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan). The intensity of γH2AX nuclear foci was quantified using Dynamic Cell Count software (Keyence).

2.5. Cell cycle analysis

Cell cycle profiles were analyzed as previously described [22]. After treatment with 5-FU, the cells were pulse-labeled with 25 μM bromodeoxyuridine (BrdU) for 10 min at 39.5 °C and fixed with 70% ethanol. The fixed samples were denatured with 2 N HCl, incubated with anti-BrdU antibody (MBL, Nagoya, Japan), and stained with Alexa488-conjugated anti-mouse IgG antibody (Invitrogen) and propidium iodide (PI). The samples were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

2.6. Detection of apoptosis using annexin V–FITC

Apoptosis was detected using an annexin V–FITC apoptosis detection kit (Sigma). Briefly, 5×10^5 cells were resuspended in 0.5 ml of 1× binding buffer (10 mM HEPES/NaOH, pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with 0.5 μg/ml of the annexin V–FITC conjugate and 2 μg/ml PI for 10 min at room temperature before FACS analysis. Annexin V–FITC-positive, PI-negative cells were counted as apoptotic cells. Experiments were done in triplicate.

2.7. Neutral comet assay

The neutral comet assay was done using a CometAssay® kit (Trevigen, Gaithersburg, MD) in accordance with the manufacturer's instructions [25]. Briefly, drug-treated cells were mixed with low melting point agarose at 37 °C and immobilized on agarose-precoated slides. After lysis, the slides were electrophoresed for 18 min at 1 V/cm in 1× TBE buffer and stained with SYBR Green. All epifluorescence images were captured using a BIOREVO BZ-9000 fluorescence microscope and each comet tail moment was calculated using CometScore™ software (TriTek, Sumerduck, VA).

3. Results

3.1. 5-FU induces γH2AX nuclear foci and Chk1-dependent DNA damage responses in chicken B lymphoma cells DT40

5-FU activates Chk1 by ATR-mediated phosphorylation of Ser345 within the C-terminal domain of Chk1, and induces S-phase arrest in HeLa cells and DT40 cells [8,9]. In HeLa cells, 5-FU also induces γH2AX, a marker of DNA damage including DSBs, although Chk1 Ser345 phosphorylation occurs before this step [8], suggesting that DNA damage is introduced after activation of the S-phase checkpoint.

When DT40 cells were exposed to increasing doses of 5-FU for 16 h, the number of cells with γH2AX nuclear foci increased in a dose-dependent manner (Fig. 1A, upper, and B). Similarly, Chk1 Ser345 phosphorylation was also induced in a dose-dependent manner (Fig. 1C), as previously described [9]. FancD2 monoubiquitination (Fig. 1C) and nuclear foci (Fig. 1A, lower), markers for ATR-dependent activation of the Fanconi anemia (FA) pathway [26–28], were also increased. The percentage of γH2AX and FancD2 foci-positive cells was greatest (>80%) at 160 μM 5-FU (Fig. 1B). In contrast, the ternary complex of TS, a marker for TS inhibition, was fully formed after treatment with 5 μM 5-FU for 16 h (Supplementary Fig. S1A). Because low 5-FU concentrations were sufficient for full TS ternary complex formation, it seems that much of the dose-dependent increase in 5-FU-induced DNA damage responses is due to misincorporation of FdUTP into DNA. This is because the cellular concentration of FdUTP, but not of dUTP, increases as the 5-FU concentration increases if TS activity is completely inhibited, even at low 5-FU concentrations. Similar dose-dependent increases in γH2AX nuclear foci (Supplementary Fig. S2A and B), FancD2 monoubiquitination and Chk1 Ser345 phosphorylation (Supplementary Fig. S2C) were also observed in the SW620 colon cancer cell line, indicating that these events are not specific to chicken DT40 cells.

When cells were cultured with a high concentration (200 μM) of 5-FU, the number of γH2AX foci-positive cells started to increase between 4 and 8 h (Fig. 1D, upper, and E). Chk1 phosphorylation (Fig. 1F) and FancD2 nuclear foci (Fig. 1D, lower, and E) were slightly increased at 4 h, and increased gradually between 4 and 16 h. In this condition, an increase in number of S-phase cells was observed at 4 h and most of the cells had accumulated in the S-phase at 8 h (Fig. 1G). At this 5-FU concentration, the ternary complex of TS was fully formed at 2 h (Supplementary Fig. S1B). Taken together, these data suggest that dTTP starvation caused by 5-FU-induced TS inhibition and the following FdUTP or dUTP misincorporation into DNA ultimately result in DNA damage associated with γH2AX foci formation and S-phase arrest.

3.2. Mutants of HRR impair 5-FU-induced Chk1 phosphorylation and exhibit high sensitivity to 5-FU

To further characterize 5-FU-induced DNA damage, we performed clonogenic survival assays using mutant DT40 cells with a defective DNA repair pathway. Mutant cells with defects in HRR (*rad54* and *brca2ΔCTD*) showed significantly higher sensitivity to 5-FU than did the wild-type cells, whereas mutants with a defect in non-homologous end-joining (*ku70*) or in the FA pathway (*fancd2*) (Fig. 2A) did not, although 5-FU induced FancD2 monoubiquitination and nuclear foci formation (Fig. 1). Consistent with the contribution of HRR to 5-FU tolerance, Rad51 and γH2AX formed colocalized nuclear foci in 5-FU-treated cells (Fig. 2B). Next, we performed neutral comet assays using wild-type DT40 cells treated with 200 μM 5-FU for 16 h to determine whether DSBs were induced or not. Surprisingly, we found no increase in tail moments in this experiment, whereas 6 h of treatment with 0.5 μg/ml mitomycin C induced significant increases in tail moments (Fig. 2C). These data suggest that the HRR pathway plays a significant role in the tolerance to 5-FU cytotoxicity, even though the major DNA damage caused by 5-FU does not include DSBs.

3.3. Chk1 inhibition enhances 5-FU cytotoxicity in wild-type and HRR-deficient cells

Our data suggest that HRR contributes to 5-FU tolerance, but not via its DSB repair activity. Recent studies have shown that HRR has multiple functions at stalled replication forks [29,30], when the ATR–Chk1 signaling pathway is activated. Moreover, Chk1 kinase plays an important role in HRR by phosphorylating Rad51 at Thr309 [31]. Next, to determine whether inhibition of Chk1 kinase and defective HRR affects 5-FU sensitivity, we performed clonogenic survival assays of wild-type, *rad54* and *brca2ΔCTD* cells in the presence of UCN-01, a Chk1 kinase inhibitor that can inhibit chicken Chk1 kinase [9]. 5-FU sensitivity in the clonogenic survival assay was significantly enhanced in wild-type cells treated with UCN-01 (Fig. 3A). When we treated wild-type cells with 20 μM 5-FU and UCN-01, we observed significant increases in γH2AX nuclear foci (Fig. 3B) and comet tail moments (Fig. 3C), which suggests that DNA DSBs were induced by 5-FU when combined with inhibition of Chk1 kinase. In HRR-deficient *rad54* and *brca2ΔCTD* cells, 5-FU sensitivity was enhanced in cells treated with UCN-01 (Fig. 3D and E). Consistently, when we treated these mutant cells with 20 μM 5-FU and UCN-01, we noted significant increases in Chk1 phosphorylation, FancD2 monoubiquitination and γH2AX, similar to those in wild-type cells (Fig. 3F). Increased γH2AX was not caused by apoptosis,

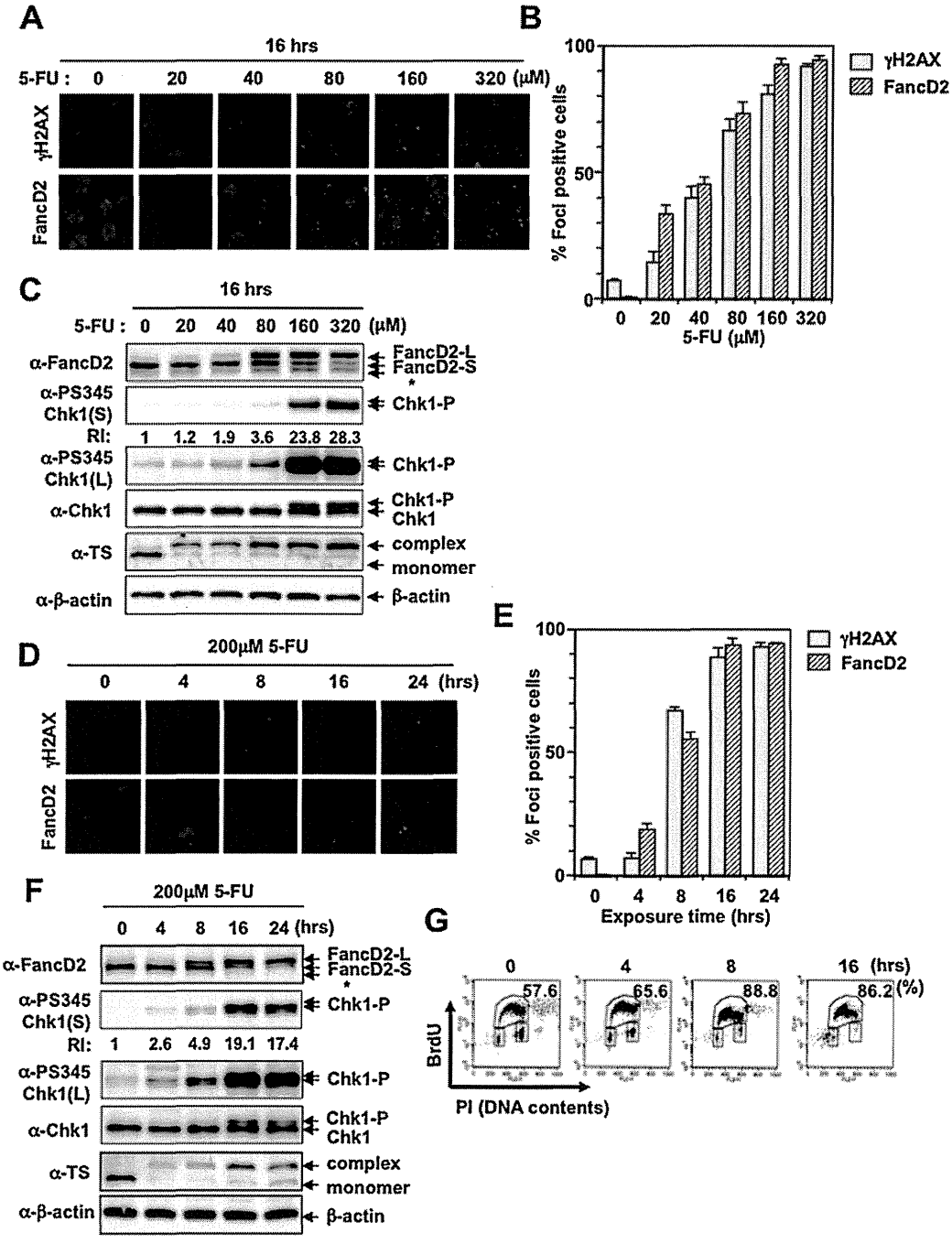

Fig. 1. Induction of Chk1 phosphorylation, and γH2AX and FancD2 nuclear foci by 5-FU. (A) Dose-dependent effects of 5-FU on FancD2 and γH2AX nuclear foci formation. Wild-type DT40 cells were treated with the indicated concentrations of 5-FU for 16 h. Nuclei were counterstained with DAPI. (B) Percentage of foci-positive cells in (A). Cells with five or more discrete foci were counted as positive. Error bars indicate standard deviation (SD) of triplicate samples. (C) Dose-dependent effects of 5-FU on FancD2 monoubiquitination and Chk1 phosphorylation. Wild-type DT40 cells were treated with the indicated concentrations of 5-FU for 16 h. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibody. S: short exposure, L: long exposure, RI: relative intensity of Chk1 Ser345 phosphorylation. (D) Time-dependent formation of FancD2 and γH2AX nuclear foci in response to 5-FU. Wild-type DT40 cells were treated with 200 μM 5-FU. Nuclei were counterstained with DAPI. (E) Percentage of foci-positive cells in (D). Cells with five or more discrete foci were counted as foci-positive. Error bars indicate SD of triplicate samples. (F) Time-dependent response of FancD2 monoubiquitination and Chk1 phosphorylation to 5-FU. Wild-type DT40 cells were treated with 200 μM 5-FU for the indicated times. Whole-cell extracts were blotted with the indicated antibody. S: short exposure, L: long exposure. (G) Analysis of BrdU incorporation in wild-type and *chk1* cells treated with 200 μM 5-FU for the indicated times. The percentage of BrdU-positive cells is shown.

because, at this concentration of 5-FU, UCN-01 only weakly affected 5-FU-induced apoptosis, which was detected as the annexin V-positive/PI-negative population (Supplementary Fig. S3C). These data suggest that inhibition of Chk1 kinase enhances 5-FU cytotoxicity by increasing 5-FU-induced DNA damage detected as γH2AX nuclear foci not only in wild-type cells but also in HRR-deficient cells.

3.4. Rad9- and Rad17-deficient cells exhibit 5-F U hypersensitivity, which coincides with impaired Chk1 phosphorylation, increased DNA damage including DSBs and increased apoptosis

Rad9 and Rad17 play critical roles in the activation of the ATR–Chk1 signaling pathway initiated by RPA-coated ssDNA [7,11].

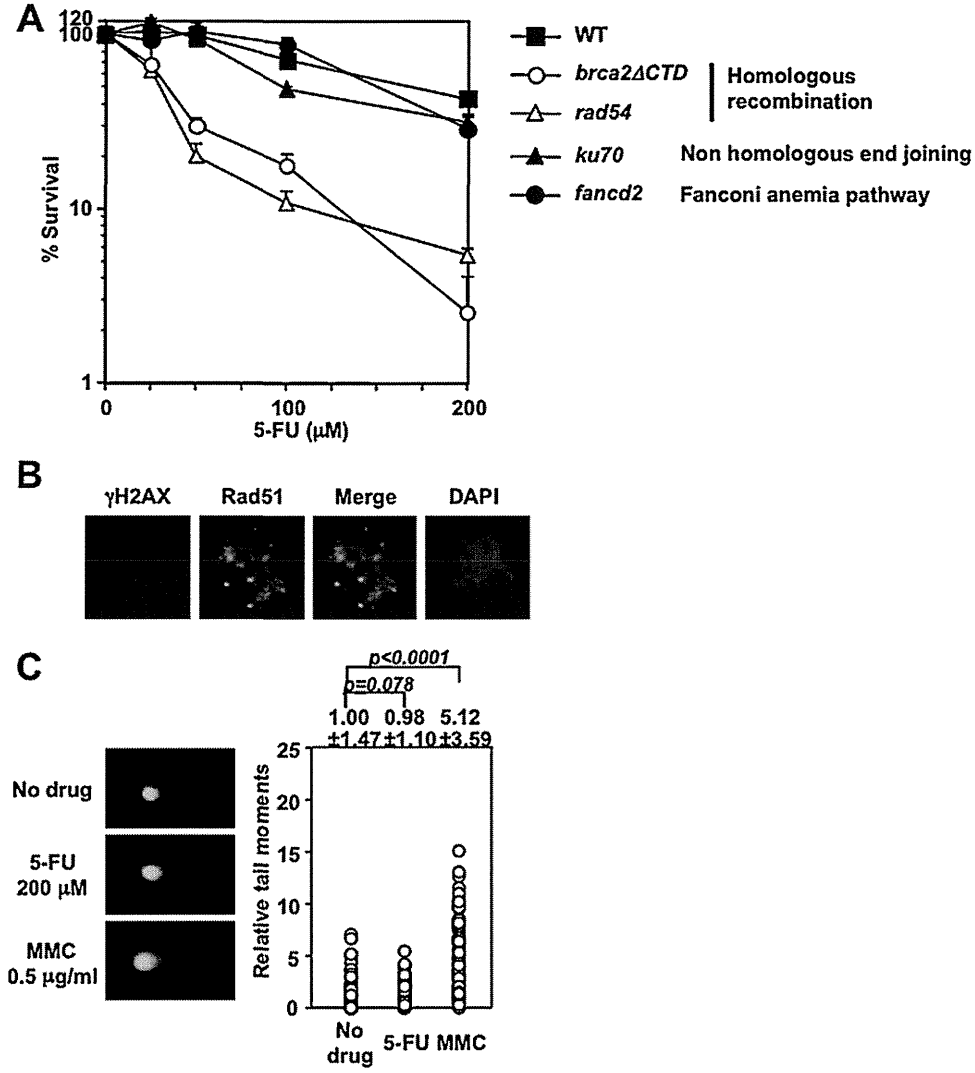

Fig. 2. Mutants of homologous recombinational repair are hypersensitive to 5-FU. (A) Clonogenic survival assay of wild-type and mutant (*brca2ΔCTD*, *rad54*, *ku70*, and *fancd2*) cells. Error bars indicate SD of triplicate samples. (B) Colocalization of γH2AX and Rad51 in 5-FU-treated cells. Wild-type cells were treated with 80 μM 5-FU for 16 h and co-immunostained with antibodies against γH2AX and Rad51. Nuclei were counterstained with DAPI. In merged images, yellow foci indicate colocalization. (C) Representative images of nuclei treated with 200 μM 5-FU and 0.5 μg/ml mitomycin C (MMC) in the neutral comet assay and relative tail moments. The mean tail moment in untreated cells was set as 1.00. *P* values were determined using the Mann–Whitney *U*-test.

To elucidate the possible involvement of Rad9 and Rad17 in the 5-FU-induced DNA damage responses and 5-FU tolerance, we analyzed the cellular response to 5-FU in *Rad9*- and *Rad17*-deficient DT40 cells (*rad9* and *rad17*).

In the clonogenic survival assay, *rad9* and *rad17* cells showed significantly greater sensitivity to 5-FU than did the wild-type cells (Fig. 4A). At the time of inoculation for the clonogenic survival assay (*i.e.*, at 16 h), 5-FU-induced Chk1 Ser345 phosphorylation was impaired in these mutant cells, particularly at high concentrations of 5-FU (80 and 200 μM) (Fig. 4B). An accumulation of cells in the S-phase was not seen following 5-FU treatment (Supplementary Fig. S4A), whereas a dose-dependent increase in the number of apoptotic cells was observed in these mutant cells (Fig. 4C and Supplementary Fig. S4B). In addition, when *rad9* and *rad17* cells were treated with 80 or 200 μM 5-FU, we detected cleaved Chk1 peptides (Fig. 4B). These Chk1 peptides were not detected when the cells were treated with 5-FU in the presence of ZVAD-fmk, a caspase inhibitor (Supplementary Fig. S5), confirming that these peptides were produced by caspase-mediated Chk1 cleavage during apoptosis [32]. When *rad9* and *rad17* cells were treated with 40 μM 5-FU, a concentration at which fewer than 20% of *rad9* or *rad17* cells underwent apoptosis, more cells were positive for γH2AX nuclear foci compared with wild-type cells (Fig. 4D and E). Furthermore, the foci showed stronger intensity compared with those of wild-type cells (Fig. 4D, bottom, and Supplementary Fig. S4C). The neutral comet assay revealed an increase in tail moments in *rad9* and *rad17* cells treated with 40 μM 5-FU, but not in wild-type cells (Fig. 4F). These data suggest that cells suffered severe DNA damage, including DSBs, and were more susceptible to apoptosis in the absence of Rad9 or Rad17.

DNA DSBs or γH2AX nuclear foci are also detected in cells undergoing apoptosis [33]. Next, we examined whether there is a kinetic difference between the emergence of DNA damage and the induction of apoptosis in *rad9* and *rad17* cells. Chk1 phosphorylation was detected in *rad9* and *rad17* cells treated with 200 μM 5-FU, but was significantly impaired (Fig. 5A). In these cells, we observed normal cell accumulation in the S-phase at 4 h, but inefficient accumulation at 8 h, and the number of BrdU-positive cells was significantly decreased after 16 h of treatment with 200 μM 5-FU (Fig. 5B). This decrease in the number of BrdU-positive cells coincided with the increase in number of apoptotic cells. Indeed, the number of apoptotic cells started to increase from 8 h (Fig. 5D) and reached ~40% after 16 h of treatment (Fig. 4C) in these mutants. At this concentration of 5-FU, the number of γH2AX nuclear

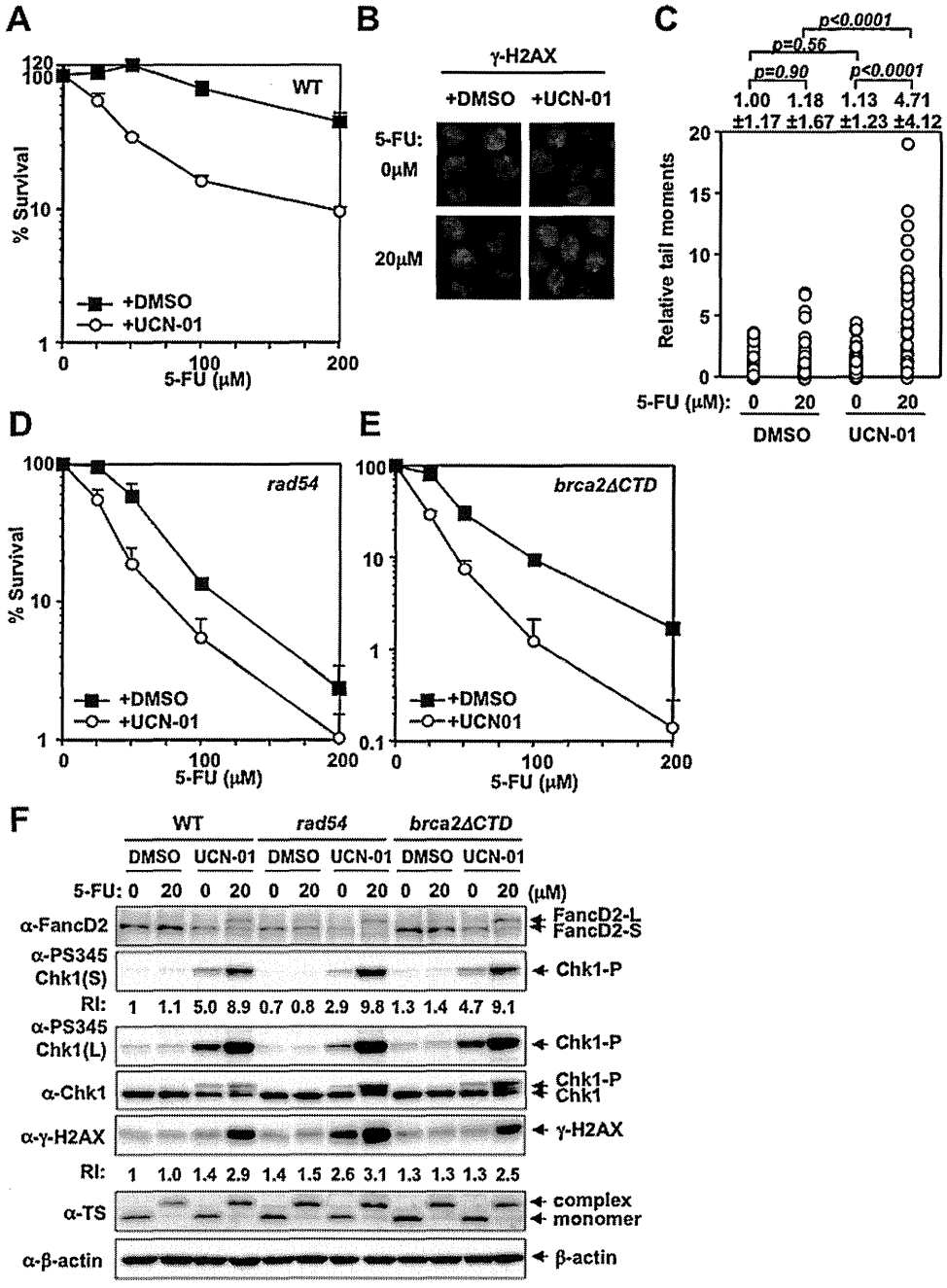

Fig. 3. Cellular response to 5-FU in the presence of UCN-01. (A) Clonogenic survival assay of wild-type cells in the presence or absence of UCN-01. Error bars indicate SD of triplicate samples. (B) Effects of UCN-01 on 5-FU-induced γH2AX nuclear foci formation. Wild-type cells were treated with 20 μM 5-FU and/or 300 nM UCN-01 for 16 h. (C) Relative tail moments determined by the neutral comet assay in 5-FU-treated wild-type cells in the presence or absence of UCN-01. The mean tail moment of DMSO-treated cells was set as 1.00. *P* values were determined using the Mann–Whitney *U*-test. (D and E) Clonogenic survival assay of *rad54* (D) and *brca2ΔCTD* (E) cells in the presence or absence of UCN-01. Error bars indicate SD of triplicate samples. (F) Effects of 5-FU and UCN-01 on FancD2 monoubiquitination, Chk1 phosphorylation and γH2AX in wild-type (WT), *rad54* and *brca2ΔCTD* cells. Cells were treated with the indicated concentrations of 5-FU for 16 h. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibody. S: short exposure, L: long exposure, RI: relative intensity of Chk1 Ser345 phosphorylation and γH2AX.

foci-positive cells started to increase from 6 h (Fig. 5C). This difference in the kinetics of γH2AX foci formation and appearance of apoptotic cells suggests that 5-FU induces DNA damage, that can be detected as γH2AX foci, followed by apoptosis in *rad9* and *rad17* cells.

3.5. UCN-01 enhances 5-FU sensitivity of Rad9- or Rad17-deficient cells

Increased DNA damage and enhanced 5-FU cytotoxicity in the absence of functional Rad9 or Rad17 are likely to be attributed to impaired 5-FU-induced Chk1 activation. However, Chk1 kinase was actually activated, albeit weakly, by 5-FU in *rad9* or *rad17* cells (Figs. 4B and 5A). To determine whether this residual phosphorylation (activation) of Chk1 kinase contributes to 5-FU tolerance in *rad9* or *rad17* cells, we performed clonogenic survival assays against 5-FU in the presence of UCN-01. UCN-01 significantly enhanced 5-FU sensitivity in both *rad9* and *rad17* cells (Fig. 6A and B), supporting the idea that Rad9/Rad17-independent activation of Chk1 kinase contributes to 5-FU tolerance. In the presence of UCN-01, there was an increase in the number of apoptotic cells, even in the absence of 5-FU (Fig. 6C). Consistently, caspase-mediated

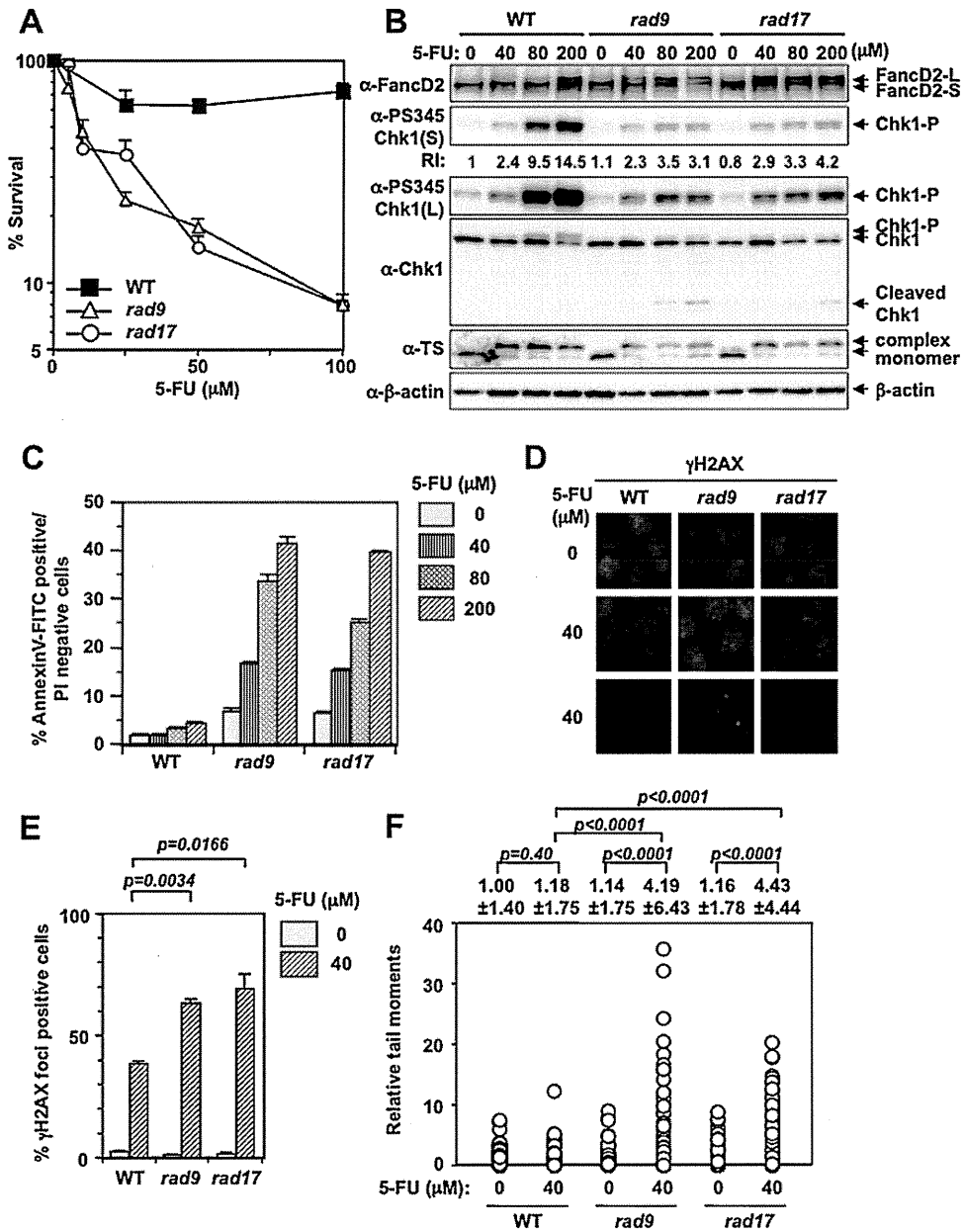

Fig. 4. 5-FU sensitivity with increased γH2AX nuclear foci and apoptosis in *rad9* and *rad17* cells. (A) Clonogenic survival assay of wild-type, *rad9* and *rad17* cells. Error bars indicate SD of triplicate samples. (B) Western blotting with the indicated antibodies of wild-type, *rad9* and *rad17* cells treated with 40, 80 or 200 μM 5-FU for 16 h. RI: relative intensity of Chk1 Ser345 phosphorylation. S: short exposure, L: long exposure. (C) 5-FU-induced apoptosis in wild-type, *rad9* and *rad17* cells. Cells were treated with 40, 80 or 200 μM 5-FU for 16 h and stained with annexin V–FITC conjugates and propidium iodide (PI). The percentage of annexin V–FITC-positive, PI-negative apoptotic cells is shown. Error bars indicate SD of triplicate samples. Representative data are shown in Supplementary Fig. S4B. (D) Nuclear γH2AX foci in cells treated with 40 μM 5-FU for 16 h. Merged images of γH2AX and DAPI (top and middle rows) and a representative image of a γH2AX foci-positive cell (bottom row) are shown. (E) Percentage of foci-positive cells in (E). Cells with five or more discrete foci were counted as foci-positive. Error bars indicate SD of triplicate samples. *P* values were determined using Student's *t* test. (F) Relative tail moments determined by the neutral comet assay in 5-FU-treated wild-type, *rad9* and *rad17* cells. The mean tail moment of untreated wild-type cells is set as 1.00. *P* values were determined using the Mann–Whitney *U*-test.

Chk1 cleavage was induced by UCN-01 alone (Supplementary Fig. S6A) and the plating efficiency of the clonogenic survival assay decreased significantly in the presence of UCN-01. The relative plating efficiency of *rad9*, *rad17* and wild-type cells treated with UCN-01 was 60.1 ± 3.6%, 66.6 ± 7.9% and 106.3 ± 7.9%, respectively. In contrast, we did not observe significant increases in γH2AX foci in cells treated with UCN-01 alone (Fig. 6D), suggesting that UCN-01-induced apoptosis was not mediated by DNA damage detected as γH2AX nuclear foci. When *rad9* or *rad17* cells were treated with a very low concentration (5 μM) of 5-FU, the number of γH2AX foci-positive cells increased significantly in the presence, but not in the absence, of UCN-01 (Fig. 6D). Taken together, these results suggest that, cells lacking functional Rad9 or Rad17 were more susceptible to 5-FU-induced DNA damage in the presence of UCN-01, and that inhibition of Chk1 kinase activity has additive effects on 5-FU cytotoxicity, even in the absence of Rad9 or Rad17.

4. Discussion

5-FU and its prodrugs have been, and will probably continue to be, the mainstay first-line treatment for major solid tumors. Although 5-FU metabolites affect DNA, RNA and *de novo* nucleotide synthesis, cytotoxicity associated with DNA damage has been a major focus of research, and BER and MMR are involved in these

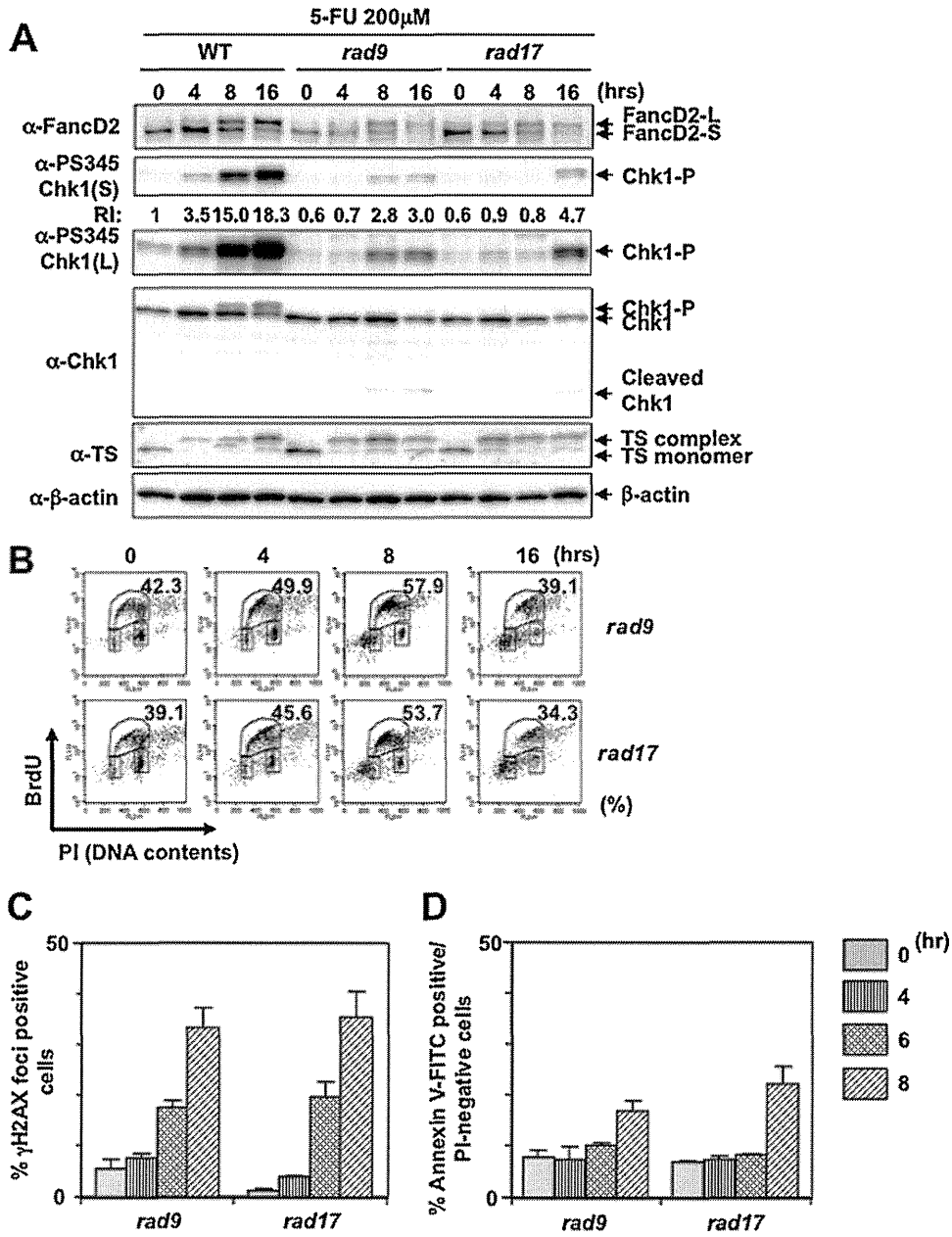

Fig. 5. Time-course analysis of Chk1 phosphorylation, γH2AX nuclear foci formation and apoptosis in response to 5-FU treatment in *rad9* and *rad17* cells. (A) Western blotting of wild-type, *rad9* and *rad17* cells treated with 200 μM 5-FU for the indicated times. S: short exposure, L: long exposure, RI: relative intensity of Chk1 Ser345 phosphorylation. (B) BrdU incorporation in *rad9* and *rad17* cells treated with 200 μM 5-FU for the indicated times. The percentage of BrdU-positive cells is shown. (C) Percentage of γH2AX nuclear foci-positive *rad9* and *rad17* cells treated with 200 μM 5-FU for the indicated times. Cells with five or more discrete foci were counted as foci-positive. (D) Percentage of annexin V-positive/PI-negative *rad9* and *rad17* cells treated with 200 μM 5-FU for the indicated times. Error bars indicate SD of triplicate samples.

events [19,34–37]. In addition, the Chk1-mediated cell cycle checkpoint also contributes to 5-FU tolerance [8,9]. From the clonogenic survival assay using DT40 mutants with defects in the DNA damage response, in addition to Chk1 mutants [9], we identified that mutants of HRR factors (*i.e.*, BRCA2 and Rad54) (Fig. 2A) and factors in the ATR–Chk1 signaling pathway (*i.e.*, Rad9 and Rad17) (Fig. 4A) were more sensitive to 5-FU than were the wild-type cells. These data suggest that (1) HRR contributes to the repair of 5-FU-induced DNA damage and protects cells from 5-FU cytotoxicity and (2) the ATR–Chk1 signaling pathway plays a critical role in 5-FU tolerance. We also found that 5-FU induced monoubiquitination and nuclear foci formation of FancD2 (Fig. 1), a marker of FA pathway activation. *FancD2*-deficient cells, however, were much less sensitive to 5-FU (Fig. 2A), even though these cells are very sensitive to DNA crosslinking agents, such as cisplatin or mitomycin C [24,38]. Monoubiquitination of FancD2 is induced by drugs that stall replication forks, such as hydroxyurea, as well as DNA damaging agents [26–28]. Monoubiquitination of FancD2 by 5-FU may be induced by stalled replication fork and may preserve the integrity of stalled replication forks, but such activity may be dispensable for the tolerance to 5-FU.

DNA damage elicited by 5-FU originates from the misincorporation of FdUTP and dUTP into DNA [2,3]. The clear dose-dependent increase in the number of γH2AX nuclear foci-positive cells (Fig. 1A) implies that incorporation of FdUTP significantly contributes to 5-FU-induced DNA damage, because the ternary complex of TS was fully formed in DT40 cells, even in those treated with very low concentrations (5 μM) of 5-FU (Supplementary Fig. S1A). In addition, these γH2AX nuclear foci were formed as the cells accumulated in the S-phase (Fig. 1D, E and G), suggesting that some cellular

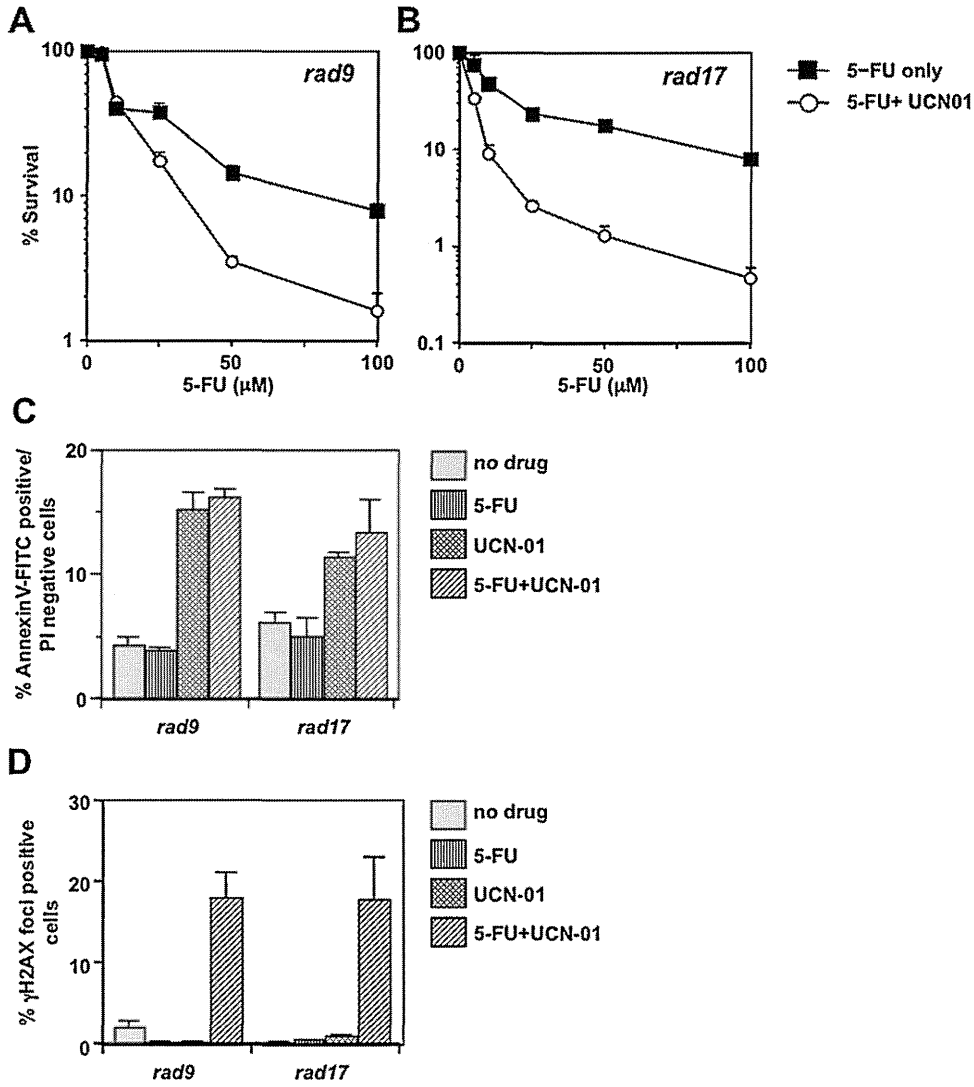

Fig. 6. The Chk1 kinase inhibitor UCN-01 potentiates 5-FU sensitivity by increasing γH2AX nuclear foci in *rad9* and *rad17* cells. (A and B) Clonogenic survival assay of *rad9* (A) and *rad17* (B) cells treated with 5-FU in the presence (open circles) or absence (closed squares) of UCN-01. Error bars indicate SD of triplicate samples. (C) Apoptosis of *rad9* and *rad17* cells treated with 5 μM 5-FU and/or 300 nM UCN-01 for 16 h. The percentage of annexin V–FITC-positive, PI-negative apoptotic cells is shown. Error bars indicate SD of triplicate samples. (D) γH2AX nuclear foci formation in *rad9* and *rad17* cells treated with 5 μM 5-FU and/or 300 nM UCN-01 for 16 h. The percentage of foci-positive cells is shown. Cells with five or more foci were counted as foci-positive. Error bars indicate SD of triplicate samples.

processes during DNA replication are involved in γH2AX nuclear foci formation. γH2AX is often considered as a marker for DSB [39,40], but accumulating evidence suggests that γH2AX can occur in the absence of DSB. For example, γH2AX arises when replication forks are stalled by UV irradiation, hydroxyurea, aphidicolin [41,42] or by nucleoside analogs, such as gemcitabine, cytarabine and troxacitabine [43,44]. A recent study also showed that γH2AX is located at the stalled replication fork before DSB formation [45]. In fact, we did not observe a significant increase in comet tail moments in the neutral comet assay in wild-type cells, even in cells treated with a high concentration (200 μM) of 5-FU (Fig. 2C). FdUTP or dUTP are misincorporated into DNA in 5-FU-treated cells, and removal of FdUTP or dUTP by BER or MMR produces ssDNA nicks or gaps, respectively [3]. These misincorporated nucleotides or ssDNA nicks or gaps may stall active replication forks and induce γH2AX nuclear foci at stalled replication forks, which are not associated with DNA DSBs.

Our data suggest that HRR factors (Rad54 and BRCA2) may contribute to 5-FU tolerance (Fig. 2A), even though 5-FU alone did not induce apparent DSBs (Fig. 2C). In addition to the critical function of HRR in DSB repair, recent studies indicate that HRR has multiple functions at stalled replication forks, including the prevention of Mre11-mediated stalled replication fork degradation by BRCA2 [29] and the promotion of replication restart by Rad51 [30]. Defects in HRR functions at stalled replication forks may enhance 5-FU cytotoxicity (Fig. 2A). Moreover, inhibition of Chk1 kinase by UCN-01 significantly enhanced 5-FU cytotoxicity in the clonogenic survival assay in wild-type cells (Fig. 3A) and in HRR-deficient cells (Fig. 3D and E). This result could be explained by the significant increase of γH2AX by 5-FU in the presence of UCN-01 similarly in wild type cells and HRR-deficient cells (Fig. 3F). This increase of γH2AX foci may reflect the excess late origin firing by Chk1 inhibition [46] and these γH2AX foci may contain DNA DSBs (Fig. 3C). In addition, since Chk1 is required for HRR [31], insufficient HRR of 5-FU-induced DNA damages in the presence of UCN-01 may contribute to the increase of γH2AX and enhancement of 5-FU cytotoxicity in wild-type cells. However, our results of HRR-deficient cells (Fig. 3D–F) suggest that HRR inhibition by UCN-01 is not the only reason for the enhancement of 5-FU cytotoxicity.

Our data showed that Rad9 and Rad17 significantly contributed to 5-FU tolerance. In *rad9* or *rad17* cells, 5-FU-induced Chk1 Ser345 phosphorylation was significantly impaired (Figs. 4B and 5A).

Anchorage of ATR–ATRIP and recruitment of TopBP1 to RPA-coated ssDNA are critical steps in Rad9/Rad17-dependent activation of the ATR–Chk1 signaling pathway in response to DNA damage or replicative stress [7]. In the absence of functional Rad9 or Rad17, activation of ATR and Chk1 was impaired and, as a result, DSBs were formed, probably because of collapsed stalled replication forks (Fig. 4F) and cells are directed to apoptosis (Figs. 4C and 5D). We also detected residual activation of the ATR–Chk1 signaling pathway in the absence of functional Rad9 and Rad17 (Figs. 4B and 5A). Rad9/Rad17-independent activation of ATR and Chk1 may initiate the S-phase checkpoint response to 5-FU, because the *rad9* and *rad17* cells accumulated normally in the S-phase after 4 h of treatment with 200 μM 5-FU (Fig. 5B). Currently, it is unclear what kind of DNA damage or which molecules are involved in this activation. ATR–ATRIP signaling with MutSα/MutLα at the DNA mismatches is one possible mechanism involved in Rad9/Rad17-independent activation of ATR and Chk1 [16,17] because MutSα preferentially binds to FdUTP:dGTP mismatches *in vitro* [19]. Alternatively, some of the repair intermediates produced by BER or MMR may activate ATR and Chk1, even in the absence of Rad9 or Rad17. In both cases, Rad9- or Rad17-independent activation of ATR and Chk1 contributed to 5-FU tolerance, since UCN-01 further enhanced 5-FU cytotoxicity in *rad9* and *rad17* cells (Fig. 6A and B).

Based on the genetic and cellular analyses of DT40 cells and their mutants, we have proposed a model explaining the relationships between 5-FU-induced DNA damage, cellular responses and cytotoxicity (Fig. 7). 5-FU-induced DNA mismatches, which are produced by misincorporation of dUTP or FdUTP into DNA, are recognized and processed by either BER or MMR machinery, resulting in the formation of ssDNA nicks or gaps. These DNA mismatches or ssDNA nicks or gaps may trigger the initial activation of the ATR–Chk1 signaling pathway in a Rad9/Rad17-independent manner, and activated Chk1 slows DNA replication. The stalled replication forks can be visualized as γH2AX nuclear foci (Fig. 1A and D) in the absence of DSBs (Fig. 2C). During this process, RPA-coated ssDNAs are generated at the stalled replication fork and induce secondary activation of the ATR–Chk1 signaling pathway in a Rad9/Rad17-dependent manner. This Rad9/Rad17-dependent activation of ATR and Chk1 may stabilize the stalled replication forks and suppress late replication origin firing. In the absence of Rad9 or Rad17, the secondary activation of the ATR–Chk1 signaling pathway is impaired (Figs. 4B and 5A), and the cells suffer from severe DNA damage, including DSBs, possibly because of the collapse of the stalled replication forks (Figs. 4D–F and 5C), ultimately directing cells to apoptosis (Figs. 4C and 5D). In addition, HRR may protect the stalled replication forks from degradation [29] or

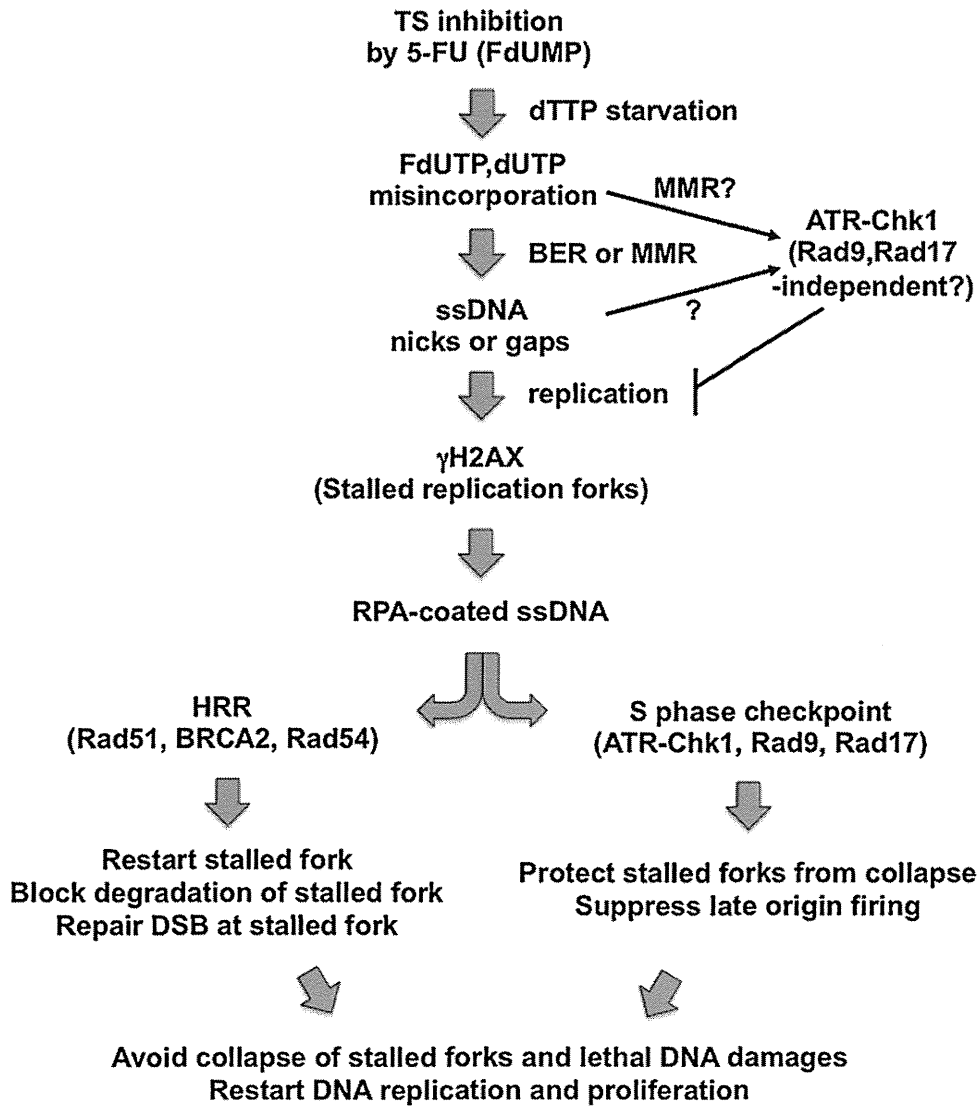

Fig. 7. Proposed model of 5-FU-induced DNA damage, cellular responses to stalled replication forks, and 5-FU cytotoxicity.

support the restart of stalled forks [47], which may contribute to 5-FU tolerance.

Our analysis of the cellular responses to 5-FU in mutant chicken DT40 cells identified several novel candidate genes that participate in the cellular response to 5-FU and which significantly influence 5-FU cytotoxicity. If our findings can be confirmed in human cancers, these gene products could offer novel molecular targets that may potentiate the antitumor effects of 5-FU-based chemotherapy.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank Dr. Shunichi Takeda for providing mutant DT40 cells, Dr. Kenshi Komatsu for providing antibodies, Ms. Naoko Katakura, Mariko Shimokawa and Akiko Seo for expert technical assistance, Ms. Satoko Hamatake for secretarial assistance and the Research Support Center, Graduate School of Medical Sciences, Kyushu University for technical support. This work was supported in part by Grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (H.K. and Y.M.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2011.11.005.

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J Epidemiol 2012;22(1):64-71
doi:10.2188/jea.JE20110059

Original Article

Genetic Polymorphisms of *XRCC1*, Alcohol Consumption, and the Risk of Colorectal Cancer in Japan

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Received May 31, 2011; accepted September 14, 2011; released online December 17, 2011

ABSTRACT

Background: X-ray cross-complementing group 1 (*XRCC1*) polymorphisms affect DNA repair capacity and may therefore be of importance in colorectal carcinogenesis. Alcohol consumption, an important risk factor for colorectal cancer, may induce carcinogenesis through DNA damage caused by the toxic effects of alcohol or its metabolites. Therefore, we examined the associations of *XRCC1* Arg399Gln, Arg280His, and Arg194Trp polymorphisms with colorectal cancer and the impact of the association between alcohol consumption and colorectal cancer risk.

Methods: This case-control study in Fukuoka, Japan including 685 cases and 778 controls. The cases were incident patients with histologically confirmed colorectal adenocarcinoma. The controls were randomly selected community subjects.

Results: The *XRCC1* 399Gln/Gln genotype was significantly associated with colorectal cancer risk (adjusted odds ratio [OR] 1.57, 95% CI 1.01–2.42; relative to 399Arg/Arg genotype). The association was strongest in individuals with high alcohol consumption. The Arg280His polymorphism modified the association between alcohol consumption and colorectal cancer risk (interaction $P = 0.049$). The OR of colorectal cancer in individuals with the 280His allele was 0.45 (95% CI 0.26–0.78) as compared with the 280Arg/Arg genotype limited to the 399Gln allele (interaction $P = 0.001$). The adjusted ORs for 399Gln/Gln-280Arg/Arg-194Arg/Arg and 399Arg/Gln-280Arg/Arg-194Arg/Trp were 1.71 (95% CI 1.02–2.87) and 1.57 (95% CI 1.05–2.33), respectively, with 399Arg/Arg-280Arg/Arg-194Arg/Arg as reference (interaction $P = 0.418$).

Conclusions: The findings are additional evidence that individuals with the *XRCC1* 399Gln/Gln genotype have an increased risk of colorectal cancer, and that *XRCC1* polymorphisms have an important role in colorectal cancer risk associated with alcohol consumption or gene-gene interaction.

Key words: *XRCC1* polymorphisms; alcohol consumption; colorectal cancer

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Abbreviations: OR, odds ratio; CI, confidence interval; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

INTRODUCTION

Colorectal cancer is one of the most common cancers in North America and Western Europe,[1,2] and its incidence has been increasing in Japan.[2] The human X-ray repair cross-complementing group 1 (*XRCC1*) gene is a DNA base-excision repair gene that has been mapped to human chromosome 19q13.[3] Human XRCC1 exhibits a number of isoenzymes, 3 of which result from known amino acid substitutions in the *XRCC1* gene, namely Arg194Trp (rs1799782), Arg280His (rs25489), and Arg399Gln (rs25487).[4] The *XRCC1* 399Gln allele has been shown to be associated with measurably reduced DNA repair capacity, as assessed by persistence of DNA adducts, increased GPA somatic mutations,[5] and elevated *p*53 mutations.[6] However, the function of the Arg194Trp and Arg280His polymorphisms remains unclear. The base-excision repair pathway is designed to remove non-bulky base adducts produced by methylation, oxidation, and reduction by ionizing radiation or oxidative damage.[7,8]

Findings regarding the associations of these polymorphisms with colorectal cancer risk have been inconsistent. For the *XRCC1* 399Gln allele, 2 previous studies showed an increased risk of colorectal cancer,[9,10] while other case-control studies showed no positive association with the that allele,[11–17] including a small study in Japan.[13] Discrepant findings have also been reported for the *XRCC1* Arg194Trp and Arg280His polymorphisms. Regarding Arg194Trp, a recent case-control study found that it was associated with a modest increase in the risk of colon cancer,[11] but this association was not observed in other studies.[9,12,14–16] Only 1[15] of several studies[11,14,15,18] showed an association between the Arg280His polymorphism and colorectal carcinogenesis.

Alcohol consumption is a known risk factor of colorectal cancer.[19,20] However, our previous study[21] showed that the *ADH1B* and *ALDH2* polymorphisms did not modify the association between alcohol consumption and colorectal cancer risk. Alcohol intake is associated with the production of reactive oxygen species—including oxygen radicals, lipid peroxidation, and acetaldehyde—which cause DNA damage that can be repaired by the DNA base-excision repair pathway.[22] Therefore, polymorphisms of the base-excision repair gene (*XRCC1*) may be a risk factor for colorectal cancer and modify the association between alcohol consumption and colorectal cancer risk.

In the present study, we examined the associations of these 3 genetic polymorphisms of the *XRCC1* gene with colorectal cancer and the impact of the association between alcohol consumption and colorectal cancer risk in Japan.

METHODS

The Fukuoka Colorectal Cancer Study was a case-control study of incident colorectal cancer patients and community control subjects residing in Fukuoka City and 3 adjacent areas. The details of the study were reported in our previous article,[23] and the methods relevant to the present analysis are described below. The study protocol was approved by the ethics committees of Kyushu University and all participating hospitals, except for 2 that did not have ethics committees. The surveys at these hospitals were conducted with the permission of the respective hospital directors.

Subjects

The cases comprised a consecutive series of patients with histologically confirmed incident colorectal adenocarcinoma who were admitted to 1 of 8 centers (2 university hospitals and 6 affiliated hospitals) for surgical treatment between October 2000 and December 2003. Other eligibility criteria included age 20 to 74 years at the time of diagnosis; residence in the study area; and no history of partial or total removal of the colorectum, familial adenomatous polyposis, or inflammatory bowel disease. Research nurses visited each hospital weekly and determined the eligibility of cases by referring to admission logs and medical records. Research nurses contacted each eligible patient with permission from an attending doctor and interviewed the patient after obtaining written informed consent. Of 1053 eligible patients, 840 (80%) participated in the interview, and 685 (65%) gave informed consent to genotyping. Reasons for nonparticipation were patient refusal ($n = 115$), refusal by the patient's physician ($n = 46$), and failure to make contact ($n = 52$).

Eligibility criteria for control subjects were the same as those for case patients, except for diagnosis of colorectal cancer and age 20 to 74 years at the time of selection. A total of 1500 persons were selected as control candidates by 2-stage random sampling from among residents living in 15 communities. Of these, individuals meeting any of the following criteria were excluded: death ($n = 7$), migration from the study area ($n = 22$), undelivered mail ($n = 44$), mental incompetence ($n = 19$), history of partial or total removal of the colorectum ($n = 21$), diagnosis of colorectal cancer after the survey ($n = 5$), no response ($n = 158$), and refusal to participate ($n = 391$). Exclusion of the first 6 categories of outcome ($n = 118$) left 1382 eligible persons, of whom 833 (60%) participated in the interview and 778 (56%) gave informed consent to genotyping.

Interview

Research nurses used a uniform questionnaire interviewed all subjects in person regarding lifestyle factors, including alcohol consumption, smoking, and physical activity. Interviews for case subjects were carried out in the hospital during admission, while those for controls were conducted mostly at public community halls or collaborating clinics. The referent time point was the date of the onset of symptoms or screening (for cases) or the time of the interview (for controls).

Habitual alcohol consumption 5 years before the referent time point was ascertained. Individuals reported the average

number of days per week that alcohol was consumed and the average amount of alcohol per day of drinking. Alcohol consumption was measured in conventional units: 1 *go* (180 ml) of *sake*, 1 large bottle (633 ml) of beer, half a *go* (90 ml) of *shochu*, 2 shots (60 ml in total) of whisky or brandy, and 2 glasses (200 ml in total) of wine were each expressed as 1 unit.

Detailed information on smoking history was elicited from individuals who had smoked cigarettes daily for 1 year or longer. Smoking history included the age at which the subject started and quit smoking, along with the number years of smoking and average number of cigarettes smoked per day for each decade of age from the second to the eighth decade. Cumulative exposure to cigarette smoking per decade was expressed as the product of the number of cigarettes smoked per day and the number of years of smoking in each decade.

Height (cm), current body weight (kg), and body weight 10 years before the study were reported. Body mass index (BMI, kg/m^2) 10 years before the study was used in the analysis because current BMI is unrelated to colorectal cancer risk.[24] Questions on physical activity elicited information on the type of job (sedentary or standing work; work involving walking, laboring, and hard laboring; and unemployment), activities in commuting and housework, and leisure-time activities 5 years before the study. Leisure-time physical activity was expressed as the product of metabolic equivalents (METs) and hours of weekly participation in each activity. Parental colorectal cancer was also elicited.

Genotyping

A 5-ml venous blood sample was taken after the interview. DNA was extracted from the buffy coat using a commercial kit (Qiagen GmbH, Hilden, Germany), and genotyping was performed by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. PCR was performed in a reaction mixture of 10 μl containing 0.5 units of Taq and 1 μl of template DNA with a concentration of 50 to 150 ng/μl. The *XRCC1* Arg194Trp and Arg399Gln genotypes were determined according to the methods described by Lunn et al.[5] Primers for the Arg194Trp (rs1799782) gene were 5′-GCC CCG TCC CAG GTA-3′ (sense) and 5′-AGC CCC AAG ACC CTT TCA CT-3′ (antisense), and primers for the Arg399Gln (rs25487) gene were 5′-TTG TGC TTT CTC TGT GTC CA-3′ (sense) and 5′-TCC TCC AGC CTT TTC TGA TA-3′ (antisense). Both the 194Arg and 399Arg alleles create *Msp*I sites. PCR products were digested with *Msp*I (10 units) for 3 hours at 37°C in a mixture of 20 μl, resulting in fragments of 292 bp and 21 bp for the 194Arg allele and 313 bp for the 194Trp allele; and 374 bp and 221 bp for the 399Arg allele and 615 bp for the 399Gln allele. Arg280His (rs25489) was determined according to the method of Yin et al.[25] 5′-CCC CAG TGG TGC TAA CCT AA-3′ (sense) and 5′-CTA CAT GAG GTG CGT GCT GT-3′ (antisense) primers were used. Twenty microliters of PCR product was digested with 10 units of *Rsa*I for 3 hours at 37°C, resulting in fragments of 246 bp and 58 bp for the 280Arg allele and 304 bp for the 280His allele. The digested PCR products were separated by electrophoresis on 3% agarose gels (NuSieve GTG, BMA, Rockland, ME, USA) and visualized with ethidium bromide.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Associations between the genetic polymorphisms and the risk of colorectal cancer were examined by multiple logistic regression analyses, which included indicator variables for sex, 10-year age class (lowest class <40 years), area of residence (Fukuoka City or adjacent areas), alcohol intake (0, 0.1–1.9, and ≥2 units/day), cigarette-years (never, <800, and ≥800), BMI 10 years before the study (<25 and ≥25 kg/m^2), type of job (sedentary, moderate, and hard work), leisure-time physical activity (0, 0.1–15.9, and ≥16 MET-hours/week), and history of parental colorectal cancer as covariates.

Adjusted odds ratios (ORs) and 95% confidence interval (CIs) were obtained from the logistic regression coefficient and the standard error for the corresponding indicator variable. Statistical significance for the interaction was tested by the likelihood ratio test, which compared logistic models with and without interaction terms. Statistical significance was defined as a 2-sided *P*-value of less than 0.05.

Deviation from the Hardy-Weinberg equilibrium was evaluated by the chi-square test with 1 degree of freedom. The linkage disequilibrium was evaluated using the expectation-maximization algorithm. The OR for a specific combined genotype was obtained using logistic regression analysis with each combined genotype as an independent variable.

RESULTS

Selected characteristics of the study subjects are summarized in Table 1. Case subjects were older than the controls and had higher prevalences of high BMI (≥25 kg/m^2) 10 years before the study, heavy alcohol intake (≥2 units/day), and parental history of colorectal cancer. In contrast, sex, area of residence, cigarette-years, type of job, and leisure-time physical activity did not substantially differ between case and control subjects.

The *XRCC1* Arg194Trp, Arg280His, and Arg399Gln genotypes were not determined in 2 controls, 1 case, and 2 controls, respectively. Genotype distributions of the Arg194Trp, Arg280His, and Arg399Gln polymorphisms in control subjects were in agreement with the Hardy-Weinberg equilibrium ($P = 0.509$ for Arg194Trp, $P = 0.180$ for Arg280His, and $P = 0.245$ for Arg399Gln). The 399Gln/Gln genotype was more frequent in cases than controls. The crude and adjusted ORs of colorectal cancer for the 399Gln/Gln genotype as compared with the 399Arg/Arg genotype were significantly higher than unity (Table 2). Regarding the Arg280His polymorphism, the 280His/His genotype was more frequent in