Mechanism of resistance to chemoradiation in p53 mutant human colon cancer

JUNICHIRO HIRO, YASUHIRO INOUE, YUJI TOIYAMA, CHIKAO MIKI and MASATO KUSUNOKI

Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Tsu, Mie 514-8507, Japan

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Abstract. To understand one of the mechanisms of resistance to chemoradiation in colon cancer cells, we investigated whether 5-fluorouracil (5-FU) mediated the expression of epidermal growth factor receptor (EGFR) and modified repair of radiation-induced DNA damage, especially in a p53 independent pathway. Cytotoxicity was determined for 5-FU combined with radiation for three colon cancer cell lines that contain mutant p53 (SW480, HT29 and WiDr), using the WST-8 colorimetric assay. EGFR and the excision repair cross complementation group 1 (ERCC1) proteins during chemoradiation were measured by Western blot analysis. SW480 cells were significantly resistant to chemoradiation compared to the other mutant p53 cell lines. The alteration of EGFR and ERCC1 proteins during chemoradiation in SW480 was apparently inversely related to that of the other radiosensitive cell lines. 5-FU-induced activation of EGFR followed by radiation in SW480 cells resulted in up-regulation of ERCC1. In contrast, 5-FU-induced degradation of EGFR followed by radiation in the other radiosensitive cell lines resulted in down-regulation of ERCC1. This suggested a complementary interaction between EGFR and ERCC1, and that 5-FU-induced EGFR activation conferred protection against radiation, through activation of DNA repair. Interaction of EGFR and ERCC1 might correlate with radiation-induced DNA damage when p53 mutant colon cancer cell lines are exposed to 5-FU followed by radiation.

Introduction

Chemoradiotherapy is now widely used for the definitive and adjuvant treatment of the majority of cancer patients. Randomized trials have shown that combination treatment improves survival compared with radiation alone in locally advanced cancers of the head and neck (1), lung (2), esophagus (3), stomach (4) and rectum (5). Over the past decades, interest in preoperative chemoradiotherapy for resectable rectal cancer has increased, since it has the advantage of enhancing locoregional control by eliminating microscopic residual disease around the primary tumor and in the draining lymphatics, with a possible positive impact on overall survival (5). Although chemoradiotherapy for resectable rectal cancer is now widely accepted because of resounding clinical successes, current practices with regard to radiation technique and chemotherapy regimen differ between countries, and even between institutions. Thus, the issue of optimal chemoradiotherapy for rectal cancer is one of the major controversies in the field of oncology. In particular, resistance to chemoradiotherapy remains an important concern, because some patients show no response and suffer side-effects despite intensive therapy.

Therefore, more accurate selection of patients who are suitable for preoperative chemoradiotherapy and understanding of resistance mechanisms should improve both oncological and surgical results. However, the mechanisms of resistance to chemoradiotherapy regimens remain largely unknown. In recent years, several approaches have been pursued toward specific modulation of defined pathways of cell death, in order to increase the therapeutic efficacy of radiation and/or reduce radiation-mediated side-effects, irrespective of concurrent chemotherapy.

The epidermal growth factor receptor (EGFR) is a 170-kDa cell surface receptor that plays a pivotal role in cell proliferation, migration and survival. Overexpression of EGFR correlates with tumor resistance to cytotoxic agents, including radiation, increased cancer relapse rate, and poor survival (6-8). Multiple lines of evidence indicate that EGFR is an important determinant of radiation response and has a radioprotective function (9). Although various intrinsic and extrinsic factors affecting radiosensitivity, including hypoxia and angiogenesis (10), DNA repair (11) and p53 gene status (12), have been reported, as well as EGFR status, we focused on the expression of EGFR and DNA repair, which may be potential mechanisms of chemo-radiosensitivity in p53 mutant human colon cancer cells.

In the present study, to understand one of the chemo-radioresistant mechanisms in colon cancer cells, we investigated whether 5-FU, which has been used extensively with radiation for colorectal cancer, mediated the expression of

Correspondence to: Dr Masato Kusunoki, Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Edobashi 2-174, Tsu, Mie 514-8507, Japan
E-mail: kusunoki@clin.medic.mie-u.ac.jp

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EGFR, and modified repair of radiation-induced DNA damage, especially via a p53-independent pathway.

Materials and methods

Cell culture. The three p53 mutant type human colon adenocarcinoma cell lines (SW480, HT29 and WiDr) were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan. These cell lines were grown in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with fetal bovine serum [10% (v/v); Gibco BRL, Tokyo, Japan], glutamine (2 mM), penicillin (1,000,000 U/l), streptomycin (100 mg/l) at 37˚C in a 5% CO_2 incubator.

Anticancer agents. 5-FU was obtained from Sigma-Aldrich, reconstituted in distilled water at appropriate concentrations, and stored at -20˚C until use.

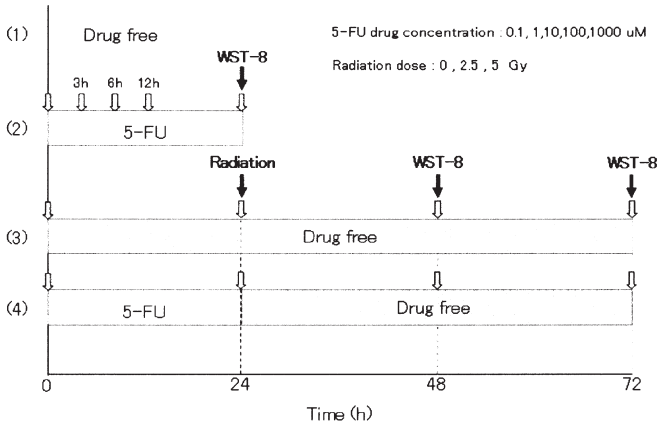
Experimental protocol. We used clinical concentrations of 5-FU as much as possible, which were chosen based on our previous reports (13,14) and drug information obtained from Kyowa Hakko Kogyo (Tokyo, Japan). The drug information on 5-FU indicated that the plasma concentration reaches 15.3 μg/ml (100 μM) after a bolus injection of 500 mg/m^2, and 0.6 μg/ml (5 μM) during continuous infusion of 5-FU (60 mg/kg/48 h). In order to elucidate the radiation effect in detail, radiation was carried out at different doses (0, 2.5 and 5 Gy). All radiation treatments were performed using the CLINAC 2100C X-ray system (Varian Oncology Services, USA) at 4 MV, using a 40-mm solid water phantom, with a dose rate of 217 cGy/min.

Chemoradiation schedules. As mentioned above, we used clinically relevant concentrations of 5-FU. Although we should ideally have considered the doubling time of each cell line before deciding the exposure time, we chose to use an exposure of 24 h for 5-FU, for experimental simplicity. The final concentrations ranged from 0.1 to 1000 μM for 5-FU. To test the cytotoxicity of each drug, each cell line was treated for 24 h with various concentrations of 5-FU. After discarding the medium that contained drug and replacing it with fresh medium, cytotoxicity was evaluated using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) colorimetric assay.

For radiation experiments, each cell line was treated with clinical concentrations of 5-FU for 24 h. After removing the drugs from the wells and refilling with fresh medium, radiation was carried out at different doses. The irradiated cells were incubated for 0 or 24 or 48 h, and cytotoxicity was evaluated using a WST-8 colorimetric assay. The drug exposure and radiation schedules are summarized in Table I.

Growth inhibition assay. Cytotoxicity was evaluated using a WST-8 colorimetric assay. WST-8 is a modification of the MTT assay, which was applied to estimation of cellular viability, using a commercially available kit (Cell Counting Kit; Dojindo Laboratories, Japan) according to the manufacturer's instructions.

Table I. Experimental protocol.



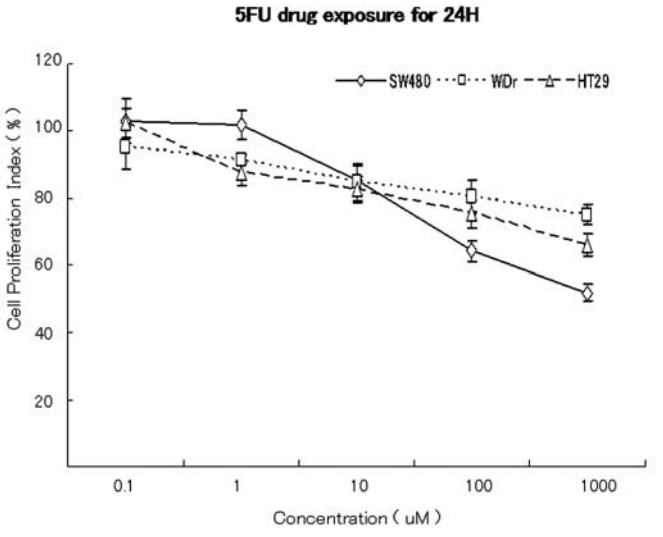


Figure 1. 5-FU dose-dependently inhibited SW480, HT29 and WiDr cell growth. 5-FU concentrations >10 μM were defined as the cytotoxic dose from each survival curve.

Western blot analysis. Protein extraction was carried out as previously described (15). The cells were homogenized in lysis buffer (Tris-buffered saline, pH 7.5, containing 2% Triton X-100) for 5 min on ice. The protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL, USA). Protein samples (20 μg) were solubilized in sample buffer by boiling and then subjecting to SDS-PAGE, followed by electrotransfer onto an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated with an appropriate primary antibody and then with peroxidase-conjugated secondary antibody. Immune complexes were visualized with the enhanced chemiluminescence Western blotting detection system (CS Analyzer version 2.0; ATTO, Japan).

The primary antibodies used were: mouse monoclonal anti- the excision repair cross complementation group 1 (ERCC1) antibody (1:250 dilution; Santa Cruz Biotechnology), a mouse monoclonal anti-EGFR antibody (1:400 dilution; Cell Signaling Technology) and mouse monoclonal anti-actin

Table II. 5-FU drug exposure for 24 h.

	0.1 μM	1 μM	10 μM	100 μM	1000 μM
SW480	102.91±6.39	101.78±4.52	85.23±4.48[a]	64.33±3.03[a]	51.98±2.34[a]
WiDr	95.05±6.45	91.50±1.14	84.47±4.21[a]	80.62±4.82[a]	75.09±2.83[a]
HT29	102.39±4.56	87.68±3.87	82.34±3.87[a]	75.21±4.22[a]	66.08±3.51[a]

Cell proliferation index ± SD (%). [a]P<0.05.

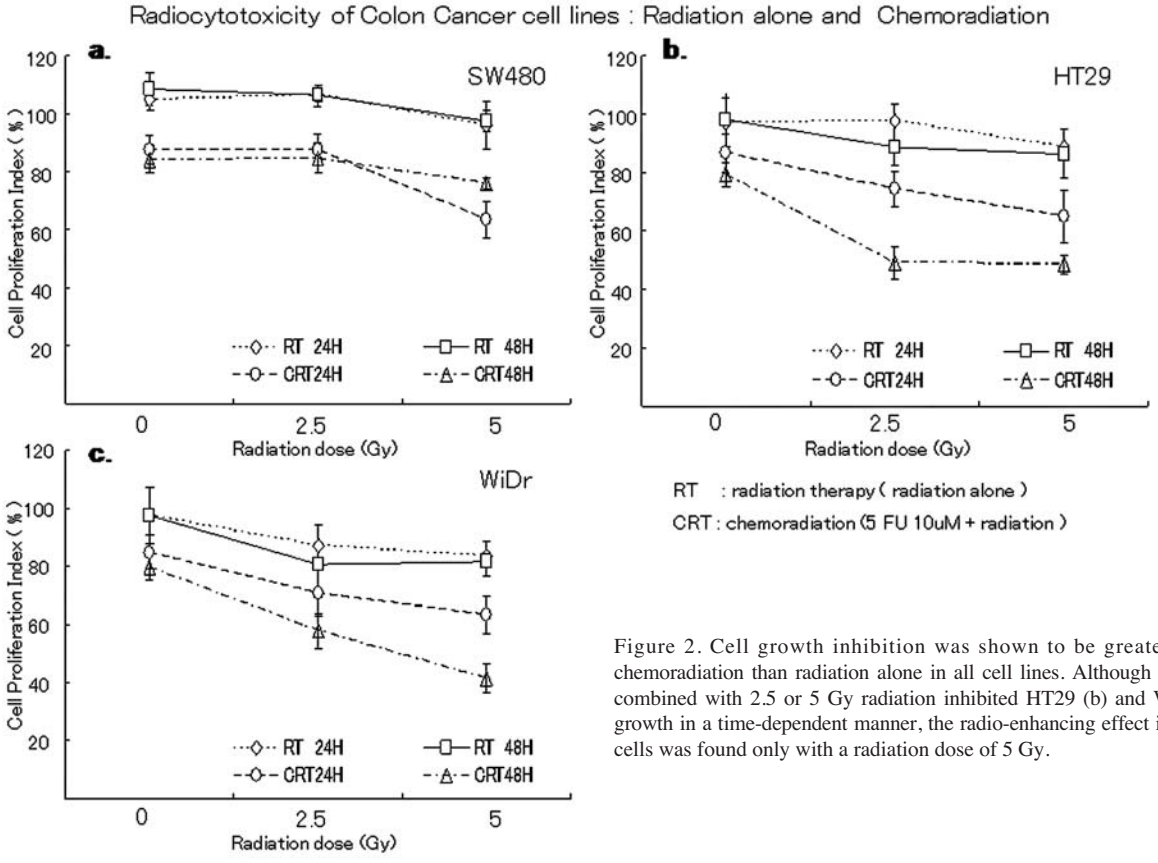


Figure 2. Cell growth inhibition was shown to be greater following chemoradiation than radiation alone in all cell lines. Although 5-FU 10 μM combined with 2.5 or 5 Gy radiation inhibited HT29 (b) and WiDr (c) cell growth in a time-dependent manner, the radio-enhancing effect in SW480 (a) cells was found only with a radiation dose of 5 Gy.

(clone C4) antibody (1:1000 dilution; ICN Biomedicals, Aurora, OH, USA). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG (Promega, Madison, WI, USA) diluted 1:1000.

Statistical analysis. The results are expressed as the means ± SD. The Mann-Whitney U test was used for comparisons between unpaired groups. P<0.05 was considered statistically significant.

Results

Cell growth inhibition of each colon cancer cell line by 5-FU. We evaluated the effects of 5-FU at 0.1-1000 μM on cell growth inhibition. The cytotoxic effects of 5-FU were assessed at 24 h after drug exposure, using the WST-8 colorimetric assay. 5-FU dose-dependently inhibited SW480, HT29 and WiDr cell growth (Fig. 1 and Table II). We defined the cytotoxic dose of 5-FU as >10 μM, from each survival curve. Therefore, the minimum cytotoxic dose of 5-FU for each cell line was 10 μM.

Cell growth inhibition by 5-FU pretreatment and radiation. SW480, HT29 and WiDr cells were treated with 5-FU at 10 μM for 24 h. After removing the fresh drug-free medium, each sample was irradiated at room temperature (22-25˚C). The radiation dose (2.5 and 5Gy) was chosen because it represented a clinically relevant radiotherapeutic dose. Cell growth inhibition was assessed by WST-8 colorimetric assay. To measure the radio-enhancing effect of 5-FU on cancer cells, we calculated the growth ratio of viable cells at 0 and 24 h, and at 0 and 48 h.

In all cell lines, growth inhibition was shown to be greater following chemoradiation than that after radiation alone. 5-FU 10 μM combined with 2.5 or 5 Gy radiation inhibited HT29 and WiDr cell growth in a time-dependent manner, however, the radio-enhancing effect in SW480 cells was found only at a radiation dose of 5 Gy (Fig. 2 and Table III).

Table III. Radiocytotoxicity of colon cancer cell lines: radiation alone and chemoradiation.

		0 Gy	2.5 Gy	5 Gy
SW480	RT 24 h	105.00±3.82	106.45±3.71	96.11±7.90
	CRT 24 h	87.49±5.08[a]	87.57±5.61[a]	63.40±6.27[a]
	RT 48 h	108.52±5.49	106.50±2.67	97.52±3.72
	CRT 48 h	85.23±4.48[a]	84.67±5.09[a]	76.00±1.81[a]
HT29	RT 24 h	97.07±8.43	97.89±5.84	88.95±5.86
	CRT 24 h	86.67±6.61[a]	74.50±5.84[a]	64.79±8.97[a]
	RT 48 h	97.97±9.07	88.53±6.38	86.22±7.65
	CRT 48 h	82.34±3.87[a]	49.24±5.43[a]	48.36±3.40[a]
WiDr	RT 24 h	97.49±9.76	87.14±7.17	83.60±4.87
	CRT 24 h	84.81±5.59[a]	70.77±8.11[a]	63.35±6.39[a]
	RT 48 h	97.49±9.76	80.90±9.64	81.88±5.40
	CRT 48 h	79.47±4.21[a]	57.64±5.88[a]	41.28±5.00[a]

Cell proliferation index ± SD (%). [a]P<0.05. RT, radiation therapy (radiation alone). CRT, chemoradiation 5-FU + radiation).

Fig. 3 shows the cytotoxicity of 2.5 Gy in all cell lines. Interestingly, SW480 was significantly resistant to chemoradiation compared to the other radioresistant cell lines. To understand the radioresistant effect of pretreatment with low cytotoxic doses of 5-FU, we searched for other possible contributory factors, including EGFR and ERCC1.

Alteration of EGFR and ERCC1 proteins during chemoradiation. Fig. 4 shows the alteration of EGFR and ERCC1 proteins during 24-h exposure to 5-FU. Exposure to 5-FU up-regulated EGFR and simultaneously down-regulated ERCC1 expression in radio-resistant SW480 cells. In contrast, exposure to 5-FU down-regulated EGFR and simultaneously up-regulated ERCC1 expression in radio-sensitive cell lines.

Additionally, the alteration of EGFR and ERCC1 proteins during chemoradiation in SW480 was apparently inversely related to that of the other radiosensitive cell lines. 5-FU-induced activation of EGFR followed by radiation in SW480 cells resulted in up-regulation of ERCC1. In contrast, 5-FU-induced degradation of EGFR followed by radiation in the other radio-sensitive cell lines resulted in down-regulation of ERCC1 (Fig. 5).

Discussion

It is well established that EGFR is one of the key players during regulation of cellular stress responses to chemotherapy and radiotherapy (16,17). EGFR not only regulates cell proliferation, but is also important for the regulation of cell survival and DNA repair (16-18). However, it is poorly understood whether alterations in EGFR and DNA repair during treatment can affect radiation sensitivity of colorectal cancer (CRC), since the optimal chemoradiation schedule for CRC is still undefined clinically.

In this study, we used three human adenocarcinoma cell lines with mutant p53, SW480, WiDr and HT29, because mutation of the p53 gene have been found in ~50% of CRC, and is associated with resistance to chemotherapy and radiation (19,20). We first attempted to establish the range of optimal concentration for 5-FU pretreatment and radiation doses based on our previous attempts to obtain radioenhancement and cell growth inhibition in three cell lines (13,14,21). SW480 cells were significantly resistant to chemoradiation

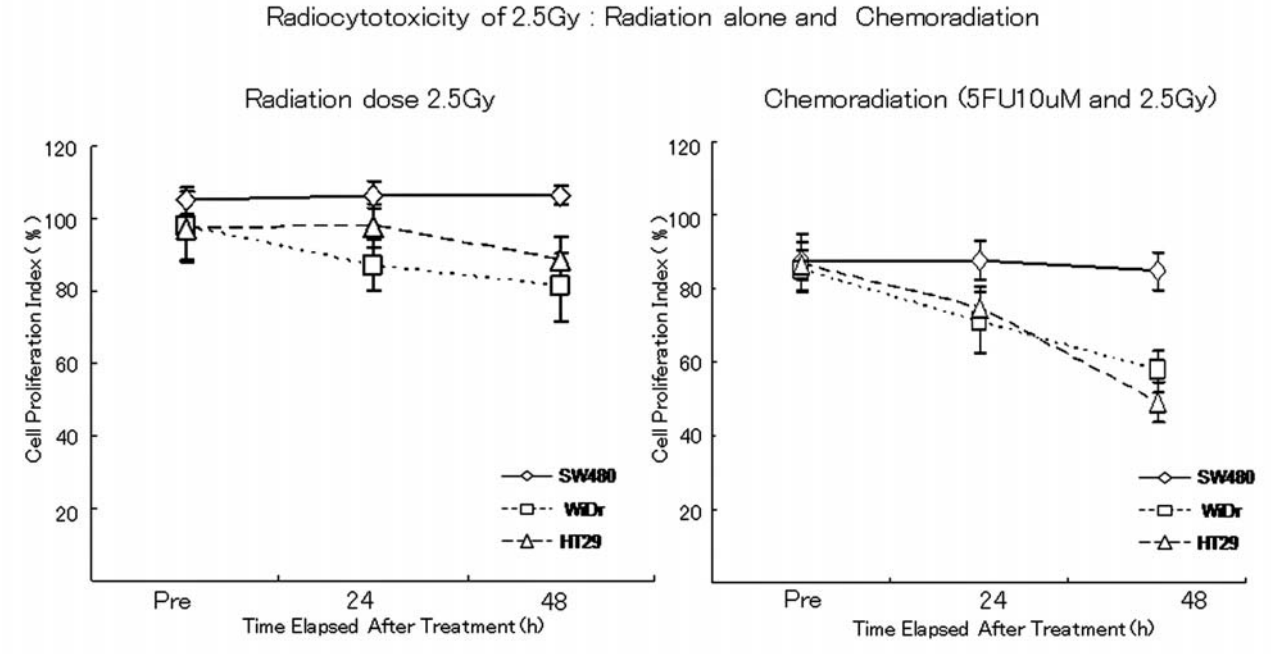


Figure 3. Despite the minimum cytotoxic dose of 5-FU and radiation, SW480 cells were significantly resistant to chemoradiation compared to other radioresistant cell lines.

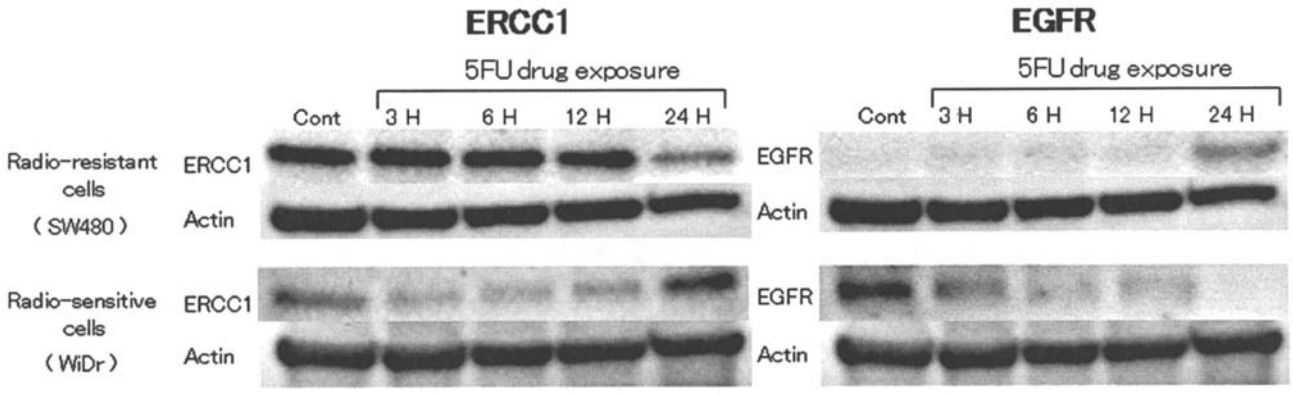


Figure 4. Exposure to 5-FU up-regulated EGFR and simultaneously down-regulated ERCC1 expression in radio-resistant SW480 cells. In contrast, exposure to 5-FU down-regulated EGFR and simultaneously up-regulated ERCC1 expression in radio-sensitive cell lines.

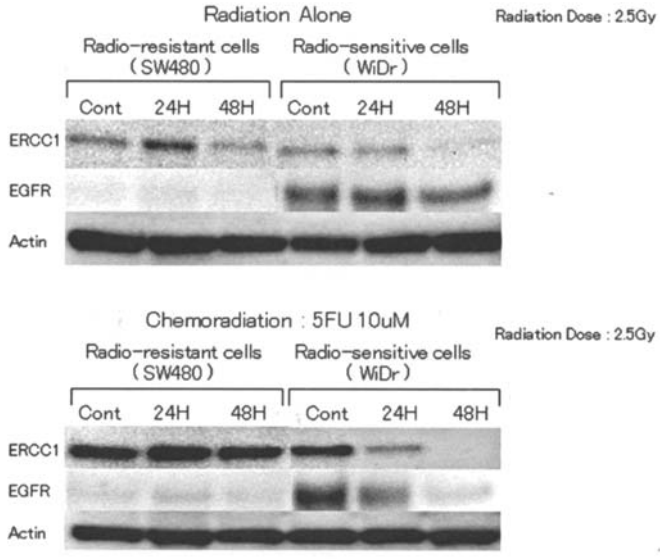


Figure 5. Alteration of EGFR and ERCC1 proteins during chemoradiation in SW480 cells was apparently inversely related to that in other radiosensitive cell lines. After chemoradiation, up-regulation of EGFR in SW480 was maintained.

compared to the other mutant p53 cell lines. To understand the radioresistant effect of pretreatment with low cytotoxic doses of 5-FU, we searched for other possible contributory factors.

Based on current evidence, EGFR-mediated radioresistance can be conceptually divided into three phases: a) an immediate early phase that involves DNA repair; b) suppression of DNA-damage-induced apoptosis before and after cell cycle arrest; and c) a tumor repopulation step that offers a proliferative advantage to tumors emerging from radiation-induced cell cycle arrest (9). Although a functional connection between EGFR and the DNA double-strand breakage repair including DNA-dependent protein kinase (DNA-PK) was reported recently (22). However, there is little information on the interaction between EGFR alterations during chemoradiation and the nucleotide excision repair pathway.

The nucleotide excision repair pathway is one of the most important that guards the integrity of the genome, which removes a wide variety of DNA lesions, including interstrand cross-links caused by cisplatin or radiation (23,24). Removal of these adducts from genomic DNA is mediated by a complex interaction of various proteins (25,26). A critical step in this process is the interaction of the product of the ERCC1 gene with those of the *Xeroderma pigmentosum* Group A (XPA) and group F (XPF) genes (27). A recent study has shown that decreased ERCC1 mRNA expression is a predictor for response to neoadjuvant chemoradiation for esophageal cancer (28). Experimental studies have demonstrated that increased ERCC1 levels are associated with removal of cisplatin-induced strand adducts, and relative cisplatin resistance (23). In addition, ERCC1-defective knockout mice are highly sensitive to DNA cross-linking agents (29). ERCC1 is associated with radiation-induced DNA damage, although this mechanism is still poorly understood (30,31).

In the present study, the alteration of EGFR and ERCC1 proteins during chemoradiation in SW480 cells was apparently inversely related to that of the other radiosensitive cell lines. We showed that 5-FU-induced activation of EGFR followed by radiation in SW480 cells resulted in up-regulation of ERCC1. In contrast, 5-FU-induced degradation of EGFR followed by radiation in radiosensitive cell lines resulted in down-regulation of ERCC1. The current results suggest that there is a complementary interaction between EGFR and ERCC1, and 5-FU-induced EGFR activation confers protection against radiation through activation of the DNA repair pathway.

In conclusion, interaction of EGFR and ERCC1 might correlate with radiation-induced DNA damage, when p53 mutant colon cancer cell lines are exposed to 5-FU followed by radiation. DNA repair may be emerging as an attractive central target in the mechanisms of chemoradiation in CRC, although further investigation is needed to elucidate the precise mechanism by which EGFR mediates the repair of radiation-induced DNA damage.

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