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Upregulation of ERCC1 and DPD expressions after oxaliplatin-based first-line chemotherapy for metastatic colorectal cancer

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BACKGROUND: The updated randomised phase 2/3 FIRIS study demonstrated the noninferiority of IRIS (irinotecan and S-1) to FOLFIRI (irinotecan, folinic acid, and 5-FU) for metastatic colorectal cancer. Meanwhile, in the subset analysis including patients who previously have undergone oxaliplatin-containing chemotherapy, the IRIS group showed longer survival than the FOLFIRI group. However, the molecular mechanism underlying this result is still unknown.

METHODS: The National Cancer Institute 60 (NCI60) cell line panel data were utilised to build the hypothesis. A total of 45 irinotecan-naive metastatic colorectal cancer patients who had undergone hepatic resection were included for the validation study. The mRNA expressions of *excision repair cross-complementing group 1* (*ERCC1*), *dihydropyrimidine dehydrogenase* (*DPD*), and *topoisomerase-1* (*TOP1*) were evaluated by quantitative RT–PCR. The expressions of ERCC1 and DPD were also evaluated by immunohistochemistry.

RESULTS: Sensitivity to oxaliplatin in 60 cell lines was significantly correlated with that of 5-FU. Resistant cells to oxaliplatin showed significantly higher *ERCC1* and *DPD* expression than sensitive cells. In validation study, *ERCC1* and *DPD* but not *TOP1* expressions in cancer cells were significantly higher in FOLFOX (oxaliplatin, folinic acid, and 5-FU)-treated patients ($N = 24$) than nontreated patients ($N = 21$). The ERCC1 and DPD protein expressions were also significantly higher in FOLFOX-treated patients.

CONCLUSION: The ERCC1 and DPD expression levels at both mRNA and protein levels were significantly higher in patients with oxaliplatin as a first-line chemotherapy than those without oxaliplatin. The IRIS regimens with the *DPD* inhibitory fluoropyrimidine may show superior activity against DPD-high tumours (e.g., tumours treated with oxaliplatin) compared with FOLFIRI.

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The combination of fluorouracil (5-FU) and folinic acid with either oxaliplatin (FOLFOX-4 and FOLFOX-6 regimens) or irinotecan (FOLFIRI and AIO regimens) has been established as the standard first-line chemotherapy for metastatic colorectal cancer (O'Neil and Goldberg, 2008). Second-line therapy for patients whose disease progresses or recurs has been investigated in several clinical studies (Cunningham *et al*, 1998; Rougier *et al*, 1998, 2002; Tournigand *et al*, 2004). Patients who are initially treated with an oxaliplatin-based regimen tend to be offered an irinotecan-based regimen as second-line therapy and vice versa. However, the basic rationale for a sequential treatment strategy has been poorly studied.

An orally administered 5-FU pro-drug, S-1, is approved for the treatment of gastric cancer, colorectal cancer, breast cancer, head and neck cancer, non-small cell lung cancer, pancreatic cancer, and hepato biliary cancer in Japan, and for gastric cancer in Europe. S-1 consists of tegafur, a pro-drug of 5-FU, 5-chloro-2,4-dihydroxypyridine (CDHP), a dihydropyrimidine dehydrogenase (*DPD*) inhibitor maintaining the serum concentration of 5-FU, and potassium oxonate, an inhibitor of orotate phosphoribosyl transferase that reduces gastrointestinal toxicities.

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We previously reported the updated results of the randomised phase 2/3 FIRIS study of 426 patients, which reconfirmed the noninferiority of IRIS (irinotecan/S-1) to FOLFIRI using progression-free survival (PFS) as the primary end point (Muro *et al*, 2010; Baba *et al*, 2011). Furthermore, we reported the pre-planned subset analysis that revealed that the median overall survival (OS) of the IRIS group in patients who previously underwent oxaliplatin-containing chemotherapy was significantly longer than that of the FOLFIRI group (adjusted HR = 0.755; 95% CI = 0.580–0.987) (Baba *et al*, 2011). Regarding this intriguing finding, Muro *et al* (2010) have speculated that S-1 might have some salvage effects in patients who previously received FOLFOX, containing oxaliplatin with bolus and infusional 5-FU. However, the mechanism underlying this interaction between the presence or absence of oxaliplatin and therapeutic effects in the FIRIS study remains unclear. The current retrospective study therefore investigated the molecular mechanisms for the superiority of IRIS to FOLFIRI in patients previously treated with oxaliplatin-based chemotherapy.

MATERIALS AND METHODS

NCI60 cell line data

The National Cancer Institute (NCI) database (http://dtp.nci.nih.gov) containing data from 60 NCI60 cell lines was used as the

source of cytotoxicity data for oxaliplatin (NSC266046), 5-FU (NSC19893), and DNA copy number. The GI_{50}, which is the concentration required to inhibit growth by 50%, was used as a parameter for cytotoxity. The DNA microarray data for gene expression were downloaded from the Genomics and Bioinformatics group website (http://discover.nci.nih.gov/). Downloaded data were processed and loaded into GeneSpring software, version 7.3 (Agilent Technologies, Santa Clara, CA, USA). Correlations were calculated using Student's *t*-tests with JMP8.0 software (SAS Institute, Tokyo, Japan).

Patient characteristics

Irinotecan-naive metastatic colorectal cancer patients, with Eastern Cooperative Oncology Group performance status (ECOG PS) 0–1, adequate organ function, and resectable liver metastases were enroled in the study. Blocks from resected tumour specimens of liver metastatic lesions were available from 24 patients who preoperatively received the FOLFOX regimen, and 21 with no prior oxaliplatin-containing chemotherapy. All patients underwent hepatic resection for colorectal liver metastasis in the Department of Gastroenterological Surgery, Kumamoto University. The study was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines. Written informed consent was obtained from all patients participating in the study. Approval of the protocol was obtained from an Independent Ethics Committee or the Institutional Review Board.

Microdissection

Representative haematoxylin and eosin-stained slides of formalin-fixed, paraffin-embedded (FFPE) blocks were reviewed by a pathologist to estimate tumour load per sample. Section slides of 10-μm thickness were then stained with nuclear fast red (Sigma-Aldrich, St Louis, MO, USA) for manual microdissection. Malignant cells were selected under microscope magnification of $\times 5$ to $\times 10$ and dissected from the slide using a scalpel as described previously (Ceppi *et al*, 2006).

Isolation of RNA and cDNA synthesis

RNA isolation from tumour tissue isolated by manual microdissection and cDNA preparation steps were accomplished as described previously (Kuramochi *et al*, 2006), with a slight modification in the extraction step using RNeasy Mini Elute spin-columns (Qiagen, Chatsworth, GA, USA).

Quantitative real-time PCR

Gene expression levels of *excision repair cross-complementing group 1* (*ERCC1*), *DPD*, and *topoisomerase-1* (*TOP1*) were determined using TaqMan real-time PCR (Life Technologies, Foster City, CA, USA) as described previously (Kuramochi *et al*, 2006). β-Actin was used (*ACTB*) as an endogenous reference gene. All genes were run on all samples in triplicate. The detection of amplified cDNA results in a cycle threshold (Ct) value, which is inversely proportional to the amount of cDNA. Universal Mix RNAs (Stratagene, La Jolla, CA, USA) were used as control calibrators on each plate. The primer sequences for *ERCC1*, *DPD*, and *ACTB* were as previously described (Schneider *et al*, 2005). The Ct was the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed level above baseline. The relative amount of tissue target mRNA standardised against the amount of *ACTB* mRNA was expressed as follows: $-\Delta Ct = -(Ct_{(target\ gene\text{-}1)} - Ct_{(\beta\text{-actin})})$. The ratio of the number of target mRNA copies to the number of *ACTB* mRNA copies was then calculated as follows: $2^{-\Delta Ct} \times K$. Here, K is a constant (Livak and Schmittgen, 2001). Contamination with genomic DNA was limited by amplifying nonreverse-transcribed RNA.

Immunohistochemistry

The FFPE tumour tissues were sliced into 4-μm sections. The tissue specimens on the slide were then deparaffinised, and endogenous peroxidase was inactivated. For ERCC1 analysis, the slides were incubated at 4 °C overnight with the primary anti-ERCC1 monoclonal antibody (Clone D-10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in a dilution of 1 : 100. For DPD analysis, the slides were incubated at 4 °C overnight with the primary anti-DPD monoclonal antibody (Clone OF-303, Taiho Pharmaceutical Co., Ltd, Tokyo, Japan) in a dilution of 1 : 100. They were then reacted with a reagent containing horseradish peroxidase-labelled polymer-bound anti-mouse IgG (EnVision + system; Dako Japan Inc., Tokyo, Japan). The chromogenic substrate used for detection was DAB (3,3′-diaminobenzidine). Slides were counterstained with haematoxylin.

Immunohistochemical data analysis

The staining intensities of ERCC1 (Kim *et al*, 2009) and DPD (Okabe *et al*, 2000) were evaluated on a scale from 0 to 2+, as described previously with slight modifications. In brief, the positive reaction for both antibodies was scored into three grades, according to the intensity of the staining: 0, 1+, and 2+. The percentages of ERCC1- and DPD-positive cells were also scored into three categories: 0 (0%), 1 (1–49%), and 2 (50–100%). The product of the intensity by percentage scores was used as the final score. The immunostained specimens were independently evaluated by two blinded investigators (HB and HO). There was close agreement (>90%) between the two investigators; in the case of any disagreement, final grading was determined by consensus.

Statistical analysis

Categorical data analysis was conducted using the χ^2 test. The GI_{50} of 5-FU and ERCC1, mRNA level of *ERCC1* and *DPD*, and immunohistochemical score of ERCC-1 and DPD were compared using Spearman's correlation coefficient. Either the Student's *t*-test or Wilcoxon test was performed to determine the differences between groups. Results were considered statistically significant at $P<0.05$. All statistical analyses were done with JMP version 8.01 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Data mining in the NCI database

The relationship between the cytotoxic effects of oxaliplatin (NSC266046) and 5-FU (NSC19893) in 60 NCI60 panel cell lines is shown in Figure 1A. The cytotoxic effects of oxaliplatin were significantly correlated with those of 5-FU (Spearman's Rho = 0.55, $P<0.0001$).

For elucidating the underlying mechanism of the correlations between oxaliplatin and 5-FU cytotoxicities, gene expression levels as determined by cDNA microarray analysis were also examined. The NCI60 panel cell lines were arbitrarily classified as oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines according to their respective GI_{50} values. The oxaliplatin-high-sensitive cell lines were those with GI_{50} values within the 15th percentile, whereas the oxaliplatin-low-sensitive cell lines were above the 85th percentile. The remaining cell lines were classified as having intermediate sensitivity.

The Student's *t*-test revealed that the gene expression level of *ERCC1* differed significantly ($P<0.05$) between oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines, as shown in

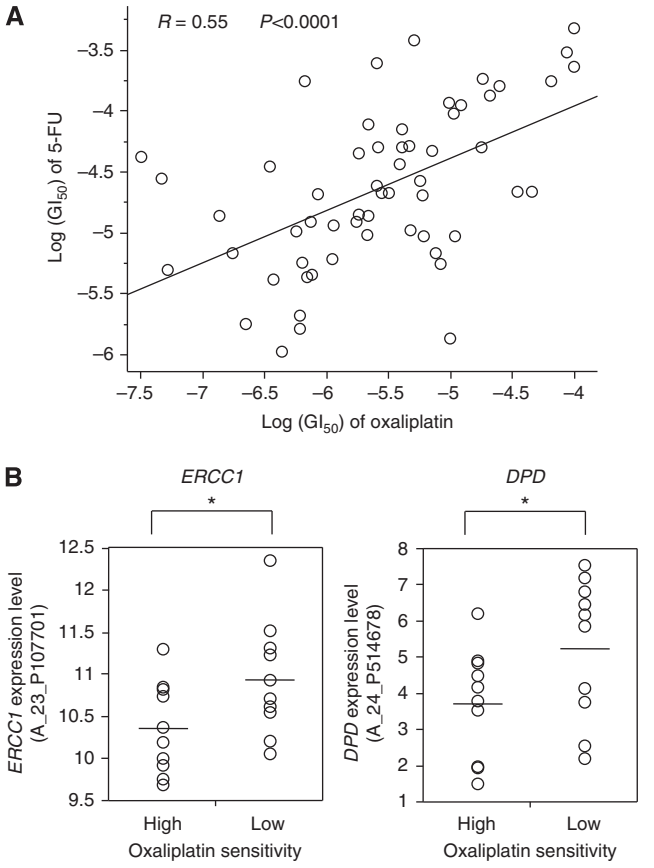

Figure 1 Oxaliplatin-resistant cells showed high *ERCC1* and *DPD* expression in *in silico* analysis. (**A**) Relationship between cytotoxic effects of oxaliplatin (NSC266046) and 5-FU (NSC19893) in 60 NCI60 panel cell lines. (**B**) Comparison of gene expression level, *ERCC1* and *DPD*, or copy number between low sensitive cells and high sensitive cells to oxaliplatin. Data expressed as $\log_2$ (per chip normalised value × 500). *$P<0.05$.

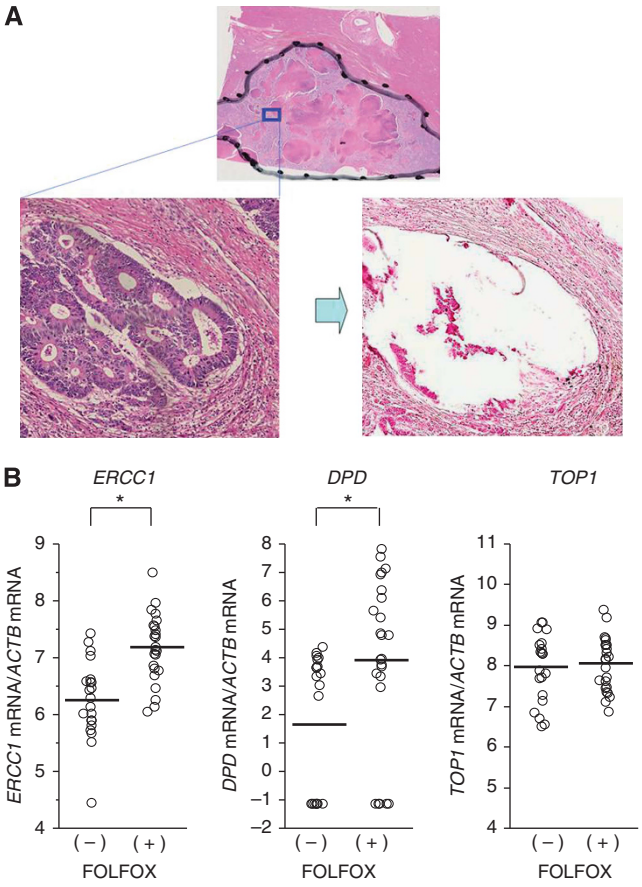

Figure 2 The *ERCC1* and *DPD* mRNAs upregulated in CRC patients with preoperative FOLFOX. (**A**) Typical slide for pathological diagnosis of FFPE tumour specimens (magnification × 2.4). Sections, 5-μm-thick, stained with haematoxylin and eosin before microdissection (magnification × 50). After staining with nuclear fast red, standard manual microdissection was performed (magnification × 50). (**B**) Comparison of gene expression levels of *ERCC1*, *DPD*, and *TOP1* in tumour cells with or without FOLFOX regimen before hepatectomy. *$P<0.001$ for ERCC1 and $P=0.005$ for DPD, respectively.

Table 1 Patient characteristics

	Oxaliplatin free, $n=21$ (%)	Oxaliplatin treated, $n=24$ (%)	*P*-value[a]
Gender, no. (%)			0.344
Male	13 (62)	18 (75)	
Female	8 (38)	6 (25)	
Age			0.715
Median, years	62	63	
Range, years	45–75	28–82	
Tumour location (%)			0.974
Proximal colon	3 (14)	3 (13)	
Distal colon	9 (43)	11 (46)	
Rectum	9 (43)	10 (42)	
Differentiation (%)			0.873
Well	10 (48)	12 (50)	
Moderate	11 (52)	12 (50)	
Prior chemotherapy (%)			—
None	19 (90)	—	
5-FU/LV	1 (5)		
S1 + CPT-11 (IRIS)	1 (5)		
mFOLFOX6	—	20 (83)	
mFOLFOX6 + bevacizumab	—	4 (17)	

Abbreviations: 5-FU/LV = fluorouracil/leucovorin; IRIS = irinotecan and S-1; mFOLFOX6 = modified FOLFOX6. [a]The *P*-values for gender were calculated using χ^2 test. The *P*-values for age, tumour location, differentiation, and prior chemotherapy were calculated using the Wilcoxon test.

Figure 1B. Interestingly, the gene expression level of *DPD* also differed significantly ($P<0.05$) between oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines (Figure 1B). Expression levels of *ERCC1* and *DPD* in oxaliplatin-low-sensitive cell lines were 1.5 and 2.9 times higher than those in high-sensitive cell lines, respectively.

Lower sensitivity to oxaliplatin was associated with a parallel increase in *ERCC1* and *DPD* expression. This finding may support that *ERCC1* influences cytotoxicity after oxaliplatin treatment. Based on the findings of recent clinical translational studies (Lentz *et al*, 2005), *ERCC1* was likely a predictive marker for colorectal cancer patients receiving oxaliplatin-containing therapy. Therefore, *ERCC1* was investigated using clinical specimens from patients who had received a first-line chemotherapy with or without oxaliplatin.

Patient characteristics

Table 1 summarises patient characteristics. The median patient age at the time of liver dissection was 62 years (range, 28–82 years). There were no significant differences in clinicopathological factors such as gender, age, tumour location, or differentiation between patients with and without a prior oxaliplatin regimen.

Gene expression level of tumour specimens

The FFPE tumour specimens resected from liver metastasis were subjected to manual microdissection to ensure that only tumour cells were dissected (Figure 2A). As shown in Figure 2B, *ERCC1* and *DPD*, but not *TOP1*, showed statistically significant higher expression in FOLFOX-treated patients ($n=24$) compared with the nontreated group ($n=21$). The mean expression level of *ERCC1* and *DPD* in those receiving the FOLFOX regimen was 1.8 and 4.9 times higher, respectively, than in patients without any prior oxaliplatin-containing chemotherapy (*ERCC1*, $P<0.0001$; *DPD*, $P=0.005$). The expression level of *ERCC1* was significantly correlated with that of *DPD* (Spearman's correlation coefficient = 0.519; $P=0.0003$).

Immunohistochemical results

The RT–PCR analysis revealed higher expression of *ERCC1* and *DPD* in FOLFOX-treated patients than nontreated patients. To confirm the protein expression levels of these genes, immunohistochemical examination was performed. The protein expression of ERCC1 (Figures 3A–C) was found in tumour cells, especially in the nucleus, whereas DPD protein expression was found in tumour cells and stromal cells (Figures 3D–F). For ERCC1, the mean (s.d.) expression was 0.48 (0.68) in patients without FOLFOX and 1.42 (1.41) with FOLFOX (Figure 3G). For DPD, the mean (s.d.) expression was 0.14 (0.36) in patients without FOLFOX and 0.79 (1.02) with FOLFOX (Figure 3G). In accordance with RT–PCR results, immunohistochemical analysis showed that protein expression of both ERCC1 and DPD was significantly higher in FOLFOX-treated patients than nontreated patients ($P=0.015$ and 0.0025, respectively; Figure 3G). Furthermore, a significant correlation between ERCC1 score and DPD score was shown (Spearman's correlation coefficient = 0.65; *P*-value <0.0001).

DISCUSSION

In the present study, gene expression levels of *ERCC1*, which were extracted by the data mining process of NCI60 screening panel data, were significantly higher in recurrent metastatic cancer cells resected from patients who had received the FOLFOX regimen than from patients with no prior oxaliplatin-containing chemotherapy. In addition, the nucleoside catabolic gene *DPD* expression level also showed significant differences between patients with and without oxaliplatin as a first-line regimen. Given that the IRIS regimens with the *DPD* inhibitory fluoropyrimidine may show superior activity against DPD-high tumours compared with FOLFIRI, our

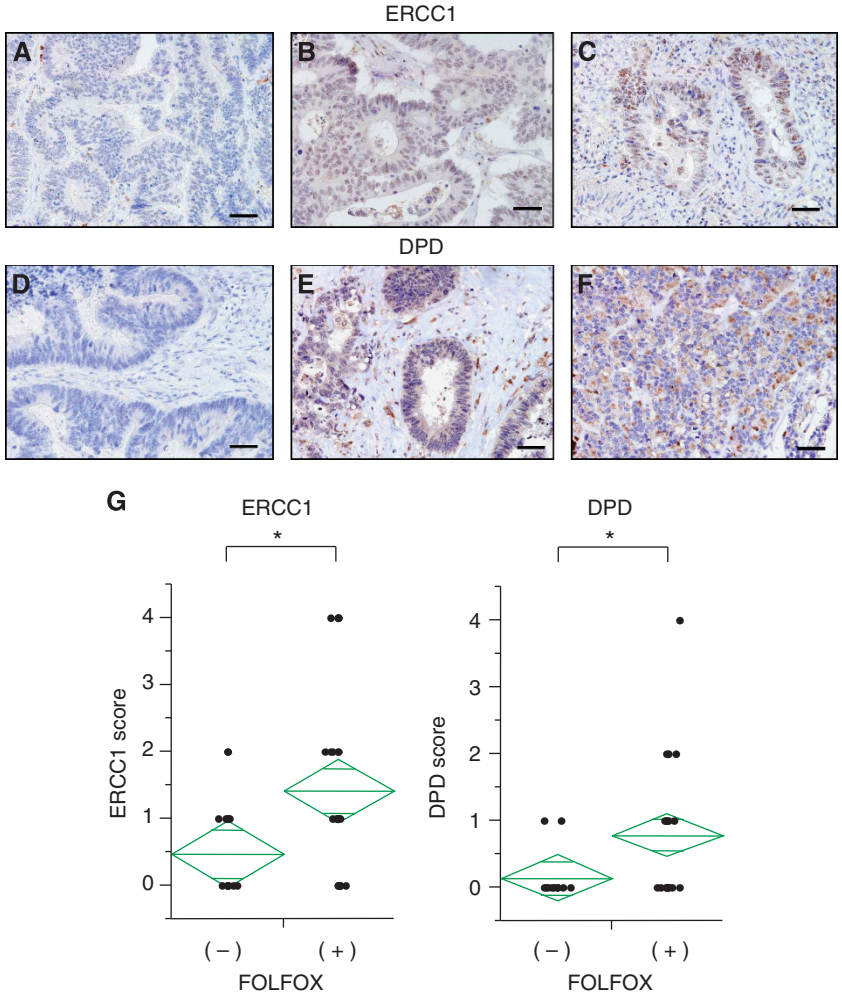

Figure 3 ERCC1 and DPD upregulated in CRC patients with preoperative FOLFOX. Representative pictures of ERCC1 and DPD in CRC patients. Cases of CRC showing weak (**A**), moderate (**B**), and strong (**C**) ERCC1 staining. Cases of CRC showing weak (**D**), moderate (**E**), and strong (**F**) DPD staining; bar = 50 μm. (**G**) The expression scores of ERCC1 and DPD were compared between patients with FOLFOX and patients without FOLFOX using Wilcoxon test. *$P=0.015$ for ERCC1 and $P=0.0025$ for DPD, respectively.

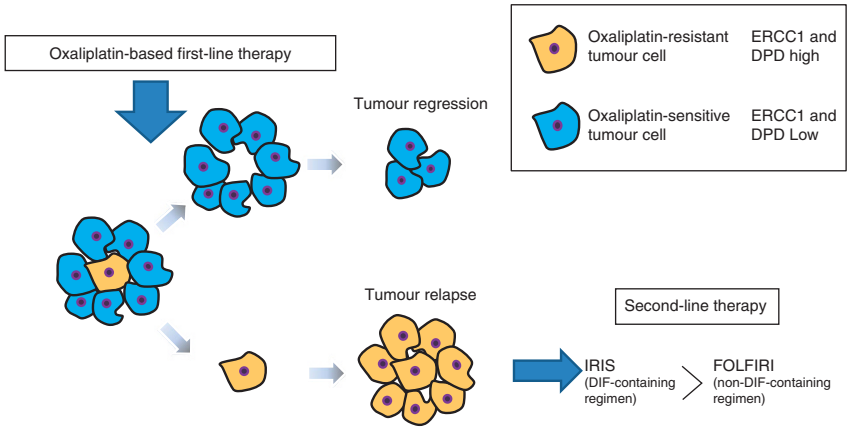

Figure 4 Hypothesis of molecular mechanism of superiority in IRIS group for prior oxaliplatin-treated patients. This study demonstrated that oxaliplatin-resistant tumour cells showed high ERCC1 and DPD, and thereby seemed to be sensitive to IRIS therapy.

findings may support the recent clinical result on the superiority of IRIS to FOLFIRI in patients previously treated with oxaliplatin-based chemotherapy.

Colon cancer is known to be a relatively heterogeneous tumour, and is characterised by a heterogenic pool of cells with distinct differentiation patterns. As an example, the *K-ras* mutation was thought to occur during early-stage tumour development; however, a recent study revealed intratumoural heterogeneity of *K-ras* mutations in 35–47% of primary colorectal carcinomas (Giaretti *et al*, 1996; Al-Mulla *et al*, 1998; Losi *et al*, 2005). Baldus *et al* (2010) also reported heterogeneity between primary tumours and lymph-node metastases in 31% (*K-ras*), 4% (*BRAF*), and 13% (*PIK3CA*) of cases. Watanabe *et al* (2011b) found intratumoral heterogeneity of *K-ras* mutations in laser-captured microdissected specimens with respect to discordant *K-ras* status between primary and metastatic colorectal tumours. Such genetic alterations, not only in *K-ras* but also in other genes, could result in intratumoral heterogeneous gene expression (Watanabe *et al*, 2011a). Recently, the concept that cancer might arise from a rare population of cells with stem cell-like properties has received support with regard to several solid tumours, including colorectal cancer (Barker *et al*, 2007; Dalerba *et al*, 2007; O'Brien *et al*, 2007; Ricci-Vitiani *et al*, 2007; Huang *et al*, 2009; Ricci-Vitiani *et al*, 2009; van der Flier *et al*, 2009). Considering the therapeutic implications of cancer stem cells, the failure of current standard therapies to eradicate tumours fully could be explained by assuming that colorectal cancer stem cells are able to survive treatments and achieve only a transitory clinical remission.

Based on our experimental results and knowledge of tumour cell biology, we propose the following hypothesis to explain why the IRIS regimen was superior to the FOLFIRI regimen for colorectal cancer patients who had been treated with oxaliplatin-based regimen. As shown in Figure 4, heterogeneous tumours were exposed to first-line oxaliplatin-containing therapy (mainly the mFOLFOX6 regimen for the FIRIS study, and partly mFOLFOX6 combined with bevacizumab). After the first-line treatment, oxaliplatin-sensitive tumour cells (i.e., *ERCC1* low; illustrated in blue in Figure 4) are killed and a small fraction of relatively oxaliplatin-resistant cells (i.e., *ERCC1* high; illustrated in yellow in Figure 4) survive, which might include cancer stem cells. In NCI60 cell line data, *ERCC1* and *DPD* gene expression is confounding; surviving cells will exhibit high *DPD* gene expression. Consequently, failure of first-line treatment might result in the proliferation of oxaliplatin-resistant tumour cells, which exhibit high levels of *DPD* gene expression. Because the IRIS (S-1/irinotecan) regimen contains S1, the *DPD* inhibitory fluoropyrimidine, it will show superior activity to FOLFIRI (5-FU/LV/irinotecan, non-*DPD* inhibitory fluoropyrimidine) against *DPD*-high tumours. This hypothesis was originally proposed when the updated results of the FIRIS study were reported at the 2011 meeting of the American Society of Clinical Oncology (ASCO) (Baba *et al*, 2011). Molecular mechanisms explaining why *ERCC1* and *DPD* gene expressions seemed to be confounding each other in cancer cells remain unclear. Recently, methylation has been recognised as an epigenetic alteration that leads to gene silencing in human cancer (Estellar, 2003). The role of aberrant methylation of the *DPD* or *ERCC1* promoter as a potential common epigenetic regulatory mechanism in tumour cells remaining after oxaliplatin-based chemotherapy warrants investigation.

A limitation of the present study was the relatively small number of patients included. Nevertheless, the phenomenon identified might be useful in selecting second-line treatments for patients who would benefit the most, and in providing a rationale for selecting therapy. To confirm our hypothesis, the study should be confirmed using an independent cohort of patients. To our knowledge, this is the first report to demonstrate a basic rationale for second-line therapy against the failures of first-line therapy containing oxaliplatin in colorectal cancer patients.

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