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 expression 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 hepatobiliary 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.

We previously reported the updated results of the randomised phase 2/3 FIRIS study of 426 patients, which confirmed 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 GI50, which is the concentration required to inhibit growth by 50%, was used as a parameter for cytotoxicity. 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 enrolled 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 ×5 to ×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-complementing group 1 (ERCC1), DPD, and topoisomerase-1 (TOPI) were determined using TaqMan real-time PCR (Life Technologies, Foster City, CA, USA) as described previously (Kuramochi et al, 2006). β-Actin (ACTB) was used 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 were preamplified using nonreverse-transcribed RNA.

**Immunohistochemistry**

The FPPE 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 χ2 test. The GI50 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 NC160 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 NC160 panel cell lines were arbitrarily classified as oxaliplatin-high-sensitive and oxaliplatin-low-sensitive cell lines according to their respective GI50 values. The oxaliplatin-high-sensitive cell lines were those with GI50 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 Schmittgen, 2001). Contamination with genomic DNA was limited by amplifying nonreverse-transcribed RNA.
Oxaliplatin causes ERCC1 and DPD upregulation in mCRC therapy

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 (NSC195983) 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 log2 (per chip normalised value number between low sensitive cells and high sensitive cells to oxaliplatin.  

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* |
|------------------------|-----------------------------|---------------------------------|----------|
| 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. *The P-values for gender were calculated using χ² test. The P-values for age, tumour location, differentiation, and prior chemotherapy were calculated using the Wilcoxon test.
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 regimen with the DPD inhibitory fluoropyrimidine may show superior activity against DPD-high tumours compared with FOLFIRI, our 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).
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. (2011) 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; Daleba 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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REFERENCES

Al-Mulla F, Going JJ, Sowden ET, Winter A, Pickford IR, Birnie GD (1998) Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 185: 130–138

Baba H, Muro K, Yasui H, Shimada Y, Tsuji A (2011) Updated results of the FIRIS study: A phase II/III trial of 5-FU/L-leucovorin/irinotecan (FOLFIRI) versus irinotecan/S-1 (IRIS) as second-line chemotherapy for metastatic colorectal cancer (mCRC). J Clin Oncol 29: 2011 (Suppl; Abstract 3562)
Baldus SE, Schafer KL, Engers R, Hartleb D, Stockeckl NH, Gabbert HE (2010) Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. *Cancer Res* 16: 790–799

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegelbarth A, Korving J, Begthel H, Peters PJ, Clevers H (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003–1007

Ceppi P, Volante M, Novello S, Rapa I, Danenberg KD, Danenberg PV, Cambieri A, Selvaggi G, Saviozzi S, Calogero R, Papotti M, Scaglioni GV (2006) ERCC1 and RPM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. *Ann Oncol* 17: 1818–1825

Cunningham D, Pyrnonen S, James RD, Punt CJ, Hickish TF, Heikkila R, Johannesen TB, Starkhammar H, Topham CA, Awad L, Jacques C, Herait P (1998) Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet* 352: 1413–1418

Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF (2007) Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 104: 10158–10163

Estellar M (2003) Relevance of DNA methylation in the management of cancer. *Lancet Oncol* 4: 351–358

Giaretti W, Monaco R, Pucic N, Papallo A, Nirgo S, Geido E (1996) Intratumor heterogeneity of K-ras2 mutations in colorectal adenocarcinomas: association with degree of DNA aneuploidy. *Am J Pathol* 149: 237–245

Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields Kim JS, Kim MA, Kim TM, Lee SH, Kim DW, Im SA, Kim TY, Kim WH, Kuramochi H, Hayashi K, Uchida K, Miyakura S, Shimizu D, Vallbohmer Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data. *Nature* 445: 106–110

O’Neill BH, Goldberg RM (2008) Innovations in chemotherapy for metastatic colorectal cancer: an update of recent clinical trials. *Oncologist* 13: 1074–1083

Okabe H, Arakawa K, Takechi T, Fukushima M (2000) Expression of recombinant human dihydropyrimidine dehydrogenase and its application to the preparation of anti-DPD antibodies for immunochemical detection. *Gann To Kagaku Ryoho* 27: 891–898

Rougier P, Lepille D, Bennouna J, Marre A, Ducreux M, Mignot I, Hua A, Mery-Mignard D (2002) Antitumour activity of three second-line treatment combinations in patients with metastatic colorectal cancer after optimal 5-FU regimen failure: a randomised, multicentre phase II study. *Ann Oncol* 13: 1558–1567

Rougier P, Van Cutsen E, Bajetta E, Niederle N, Possinger K, Labianca R, Navarro M, Morant R, Bleiberg H, Wils J, Awad L, Herait P, Jacques C (1998) Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. *Lancet* 352: 1407–1412

Schneider S, Uchida K, Brabender J, Baldus SE, Yoshim N, Danenberg KD, Salonga D, Chen P, Tsao-Wei D, Groschen S, Hoeckler AH, Schneider PM, Danenberg PV (2005) Downregulation of TS, DPD, ERCC1, GST-Pi, EGFR, and HER2 gene expression after neoadjuvant three-modality treatment in patients with esophageal cancer. *J Am Coll Surg* 200: 336–344

Tournigand C, Andre T, Achille E, Lledo G, Flech M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colini P, Louvet C, de Gramont A (2004) FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 22: 229–237

van der Flier LG, Haegelbarth A, Stange DE, van de Wetering M, Clevers H (2009) OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 137: 15–17

Watanabe T, Kobunai T, Yamamoto Y, Matsuda K, Ishiihara S, Nozawa K, Linuma H, Ikeuchi H, Eshima K (2011a) Differential gene expression signatures between colorectal cancers with and without KRAS mutations: crosstalk between the KRAS pathway and other signalling pathways. *Eur J Cancer* 47: 1946–1954

Watanabe T, Kobunai T, Yamamoto Y, Matsuda K, Ishiihara S, Nozawa K, Linuma H, Shibuuya H, Eshima K (2011b) Heterogeneity of KRAS status may explain the subset of discordant KRAS status between primary and metastatic colorectal cancer. *Dis Colon Rectum* 54: 1170–1178

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