研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅲ. 研究成果の刊行物・別刷

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Gastric Cancer Eastern Experience

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• Gastric cancer surgery • D2 dissection • Stage migration
• Quality of lymphadenectomy • Quality of postoperative care
• Splenectomy

GUIDELINES FOR THE STANDARD TREATMENT OF GASTRIC CANCER

Several guidelines are used for cancer therapy throughout the world. In the Japan Gastric Cancer Association (JGCA) guideline, standard surgery for T2 to T4 curable gastric cancer is defined as more than two-thirds gastrectomy with D2 dissection.[1] In the 2010 European Society of Medical Oncology's guideline, the standard surgery for curable gastric cancer is the D2 gastrectomy.[2] Of note, this is the first time this society has clearly advocated for the D2 approach. The National Comprehensive Cancer Network (NCCN) guidelines, commonly followed in the United States, recommend that gastric resections include regional lymphadenectomy to include the perigastric lymph nodes (D1) and those along the named vessels of the celiac axis (D2), with a goal of examining at least 15 or more lymph nodes.[3]

STAGE-SPECIFIC RESULTS OF RESECTED GASTRIC CANCER IN THE WEST AND EAST

The JGCA-maintained registry analyzed a total of 11,261 patients who underwent gastric resection in 2001.[4] The 5-year overall survival (OS) by UICC TNM stage (sixth version) was as follows: stage IA, 91.8%; stage IB, 84.6%; stage II, 70.5%; stage IIIA, 46.6%; stage IIIB, 29.9%; stage IV, 16.6%. Although the standard treatment at that time was surgery alone[5], an unknown proportion of those undergoing surgery may also have received adjuvant treatment either through enrollment into clinical trials or by doctor's or patient's choice.

Another available source of information regarding gastric cancer survival is obtained through single-institution reporting. Five-year OS after a total gastrectomy of 881 patients undergoing a total gastrectomy between 1995 and 2001 at Asan Medical Center, Korea, was 94.6%, 90.8%, 76.7%, 55.7%, 41.3%, and 15.4% for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[6] From another Korean institution, National Seoul University Hospital, the results of 10,783 consecutive patients who were surgically treated

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between 1970 and 1996 were reported. Five-year OS was 92.9%, 84.2%, 69.3%, 45.8%, 29.6%, and 9.2% for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[7] Differences in these results seem attributable mainly to the period of inclusion and improvement over time. Selection bias hampers straight comparison with nationwide registry.

The nationwide results of the United States by the National Cancer Data Base (NCDB) were reported for the cohort treated between 1985 and 1996.[8] Stage-specific OS was 78%, 58%, 34%, 20%, 8%, and 7% for stage IA, IB, II, IIIA, IIIB, and IV, respectively (**Table 1**). More recent data, after the results of Intergroup study 0116 (INT 0116), have yet to be published in medical journals. According to the report by Enestvedt and colleagues,[9] 36.8% of patients surgically staged from IB to III underwent adjuvant chemoradiotherapy after gastric resection between 2001 and 2006 in the state of Oregon. Stage-specific 5-year OS was approximately 13%, 13%, and 5% for stage IB, II, and III, respectively. These results are unacceptably poor, explained by the extremely low percentage of proper adjuvant treatment, correct staging, or adequate surgery. With this kind of data base it is not easy to obtain the precise details of patients' background, and comparison is not easy.

OVERALL SURVIVAL IN VARIOUS CLINICAL TRIALS IN THE WEST AND EAST

To know exactly the stage-specific OS by surgery alone, the most reliable way is to analyze the results of the surgery-alone arm of clinical trials that have evaluated some kind of new treatment in comparison with a surgery-alone arm as control. Since 2007, when the results of INT-0116,[10] the MAGIC trial,[11] and ACTS-GC[12] became available, it has become difficult to carry out a randomized controlled trial (RCT) having surgery alone as control.

In Japan the results of the surgery-alone arm of the ACTS-GC study, in which 1059 patients were enrolled, are available. In this trial, only stage II and IIIA/B by the Japanese classification were included. These patients can be restaged by UICC TNM classification. Some patients in stage III in the Japanese classification were classified as stage IV by TNM classification. Five-year OS was 70.8%, 56.2%, 40.1%, and 42.7% for UICC stage II, IIIA, IIIB, and IV, respectively in the surgery-only group.[13] In the Dutch Gastric Cancer Study, the 5-year OS was 81%, 61%, 42%, 28%, 13%, and 28%, for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[14] Although the Italian Gastric Cancer Study was a phase 2 study, they reported stage-specific survival due to a larger number of patients included.[15] As shown in **Table 2**, their results are somewhere between those of the ACTS-GC and the Dutch study.

Table 1
Stage-specific 5-year overall survival rate (%) of cancer registries and a large case series

	JGCA Registry	SNUH	NCDB
Period	2001	1970–1996	1985–1996
Stage IA	91.8	92.9	78
Stage IB	84.6	84.2	58
Stage II	70.5	69.3	34
Stage IIIA	46.6	45.8	20
Stage IIIB	29.9	29.6	8
Stage IV	16.6	9.2	7
Total patients	11261	10783	49756

Abbreviations: JGCA, Japan Gastric Cancer Association; NCDB, National Cancer Data Base; SNUH, Seoul National University Hospital.

Table 2
Stage-specific 5-year overall survival rate (%) of D2 surgery in clinical trials

	ACTS-GC[13]	Dutch D1 vs D2[14]	Italian P2[15]
Stage IA		81 (69)	95.0 (53)
Stage IB		61 (64)	87.5 (22)
Stage II	70.2 (278)	42 (66)	57.5 (31)
Stage IIIA	56.2 (153)	28 (72)	42.5 (37)
Stage IIIB	40.1 (53)	13 (39)	22.5 (25)
Stage IV	42.7 (35)	28 (18)	2.5 (23)
Total patients	519	328	191

Numbers in parentheses show number of patients for each stage.
Abbreviation: ACTS-GC, Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer.

In other clinical trials,[16–19] stage-specific OS cannot be obtained in publications but they would not be reliable, if available, because of the small numbers in each stage in these trials as compared with the ACTS-GC. Careful comparison of the patients' background may suggest some difference in these results. **Table 3** shows the background of the patients enrolled in the surgery-alone arm of these studies. Compared with the results of Western trials, much better OS are shown in Japanese trials (see **Table 3**).

STAGE MIGRATION: FACT AND SOURCE OF MIGRATION

Stage migration is a hampering factor when trying to compare the stage-specific results of different countries where the accuracy of staging is different. Wider lymph node dissection and more accurate lymph retrieval from the specimen result in more accurate staging, which in turn results in better stage-specific survival. Bunt and colleagues[20] evaluated the effect of stage migration in the Dutch study where D1 and D2 dissection were compared. If the patients who underwent D2 dissection were restaged abandoning the information about N2 level, 72 of 214 (34%) would have a different stage due to stage migration. Using the reported Japanese stage-specific survival results, calculated stage-specific survival by D2 staging is better in each stage than that of calculated stage-specific survival if N2 information is not used for staging. Especially in stage IIIA and IIIB, as much as 15% difference could be expected between these two staging systems. In the Japan Clinical Oncology Group (JCOG) study 9501 where D2 and D2+ para-aortic node dissection were compared, similar stage migration was observed. However, the incidence of para-aortic node metastasis (8.8%) is much smaller than that of N2 nodes, therefore only 8.5% of the entire patient cohort who underwent D3 dissection could have been restaged by abandoning the N3 information.[21]

In the Dutch study it was found that not only the extent of nodal dissection but also the way of retrieving nodes and the effort of pathologists resulted in stage migration.[22] Similarly, how the resected stomach is examined may be a source of stage migration. If the deepest part of the region is not histologically examined, earlier T stage would be attributed to these lesions.

SPLENECTOMY

In both the Dutch and the Medical Research Council (MRC) study comparing D1 with D2 surgery, splenectomy was found to be more relevant than D2 itself, due to higher

Table 3
Comparison of patients' characteristics, background, treatment, and 5-year overall survival in the surgery-alone arm of clinical trials

	JCOG 9206-2[16]	JCOG 9501[17]	INT-0116[10]	MAGIC[11]	EORCT 40954[18]	FNCLCC/FFCD[19]
No. of patients	133	523	275	253 (204)	72 (68)	110 (98)
Tumor location (%)						
L/M/U/W	39/44/37/12	217/206/100/0	154/69/50/0	NA	15/18/39	NA
Histological type (%)						
Dif/undif	43/88	204/316	77/128/70	NA	39/33	NA
pT stage (1/2/3/4)	2/39/88/4	23/257/230/13	22/63/168/22	16/55/106/16	4/30/24/7	27///58[a]
% pT3/4	69%	46%	65%	63%	48%	68%
pN (±)	101/32	348/175	231/44	114/42	52/13	68/17
% Node positive	76%	67%	84%	73%	80%	80%
Median size	5.5	5.5	NA	5.0	NA	NA
Surgery <D2/≧D2 (%)	0/132	0/523	254/20	70/96	5/63	NA
R0 resection	100%	100%	100%?	66%	67%	74%
5-Year OS	61%	70%	~25%	23%	~50%	24%

Abbreviations: Dif, differentiated; L/M/U/W, Distal part/Middle part/Proximal part/Whole stomach; NA, not available; OS, overall survival; undif, undifferentiated.
[a] T1 + 2///T3 + 4: numbers of T1 and T2 versus T3 and T4.

postoperative mortality.[23,24] In these trials, the protocol required the surgeons to carry out a splenopancreatectomy in case of a total gastrectomy in the D2 arm. Therefore, the majority of those who underwent total gastrectomy received splenectomy and distal pancreatectomy. Because of misunderstanding of the Japanese classification and definition of D category, even some patients who underwent a distal gastrectomy received splenectomy in these trials, which resulted in high mortality due to remnant stomach necrosis.[23]

Moreover, the worse prognosis of the D2 group was attributed to splenectomy in MRC trials comparing two groups of patients who underwent splenectomy or not.[25] However, it is known that prognosis of tumors located in the upper part of the stomach is worse than that of distally located tumors. The larger the tumor, the more frequently they require a total gastrectomy. These factors, biology of proximal tumor and size of tumors, seem to strongly affect the survival results. To avoid such bias, only an RCT comparing a total gastrectomy with and without splenectomy can provide a proper conclusion to this question. The JCOG performed an RCT to evaluate the noninferiority of spleen-preserving total gastrectomy to a pancreas-preserving total gastrectomy with splenectomy for patients who had T2 or deeper tumors in the proximal part of the stomach, requiring a total gastrectomy.[26] Sano and colleagues[27] reported more blood loss and higher morbidity after splenectomy, but no difference in mortality in experienced surgeons' hands. Long-term results are awaited.

IMPACT OF D2 DISSECTION ON THE RESULTS OF ADJUVANT TREATMENT

In the INT-0116 study, subgroup analysis by extent of lymphadenectomy revealed that the effect of adjuvant chemoradiation depends on the type of lymphadenectomy. Due to the limited number of those undergoing D2 dissection in this study, interaction between treatment effect and type of lymphadenectomy was not statistically significant, but those with D2 dissection did not show any benefit of adjuvant chemoradiation. These results were later transformed into the correlation between Maruyama Index (a computer program–based probability calculation of nodal residual disease) and the survival results of the patients in this study.[28] Dikken and colleagues[29] reported the influence of the extent of lymphadenectomy on the pattern of recurrence and OS in comparison with chemoradiotherapy. The investigators suggested that effect of chemoradiotherapy depends on type of lymphadenectomy, and that postoperative adjuvant chemoradiotherapy might compensate nonradical surgery for better local control.

Historically only two pivotal studies were able to show the benefit of adjuvant chemotherapy, the ACTS-GC study[12] and the CLASSIC study.[30] In these studies, all patients underwent D2 dissection as local control. The effect of radiotherapy added to adjuvant chemotherapy is being tested in two clinical trials.[31] The CRITICS trial is a European study launched in the Netherlands, wherein the effect of postoperative chemoradiotherapy (capecitabine + cisplatin with 45 Gy radiation) is compared with postoperative chemotherapy alone in the course of European standard perioperative treatment (preoperative chemotherapy comprising 3 courses of epirubicin + cisplatin + capecitabine and D1+ surgery followed by postoperative chemotherapy [same as the preoperative one]). This study is still open for accrual.[31] Another study is the ARTIST trial, a Korean single-institutional study, which compares postoperative adjuvant therapy by capecitabine + cisplatin with or without simultaneous radiotherapy. All patients should undergo D2 dissection. Four hundred and fifty-eight patients were enrolled between October 2004 and April 2008, and the short-term results, mainly concerning the safety profile, were reported in ASCO-GI 2009.[32] The final results are yet to be reported.

SUMMARY

In the East, D2 dissection shows much better results than less extended surgery followed by adjuvant treatment. Adjuvant chemotherapy without radiotherapy show significantly better survival results than surgery alone only when D2 dissection is applied. Without good local control, including regional lymph node metastasis, cure rate cannot be high.

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"...for several reasons, these two 'positive' studies will have no impact on treatment in the near future"

bevacizumab; in the GeparQuinto study, ER-negative tumors received the lion's share of the gain. As the investigators acknowledged in their discussions, there is no easy way to reconcile these disparate findings.

The large size of these trials and the importance of these groups mean that NSABP B-40 and GeparQuinto are significant test cases for the question: can neoadjuvant therapy define new standards of care in the absence of adjuvant data? Because of the relative speed and efficiency of neoadjuvant studies, and because each patient generates an informative study end point in the short term, neoadjuvant trials have been proffered as a way to accelerate drug discovery and approval in breast cancer. But, for several reasons, these two 'positive' studies will have no impact on treatment in the near future.

First, consider the end point of pCR within the breast. Although pCR has been shown to be a prognostic marker for longer-term disease-free survival (DFS),[6,7] the relationship between a step-wise improvement in pCR and any subsequent clinical gains is unclear. How much gain would a 4–6% absolute improvement in pCR—as seen in these collective experiences—translate into with respect to DFS or overall survival? No one knows. Meanwhile, the clinically assessable end points that might matter to patients, the toxicity of the experience and the chance at breast conservation, were either worse or unchanged in these two trials.[3,4]

Second, the focus on pCR within the breast may prove too narrow a surrogate. Studies have shown that even among women with pCR in the breast, the presence of residual cancer in the lymph nodes is a powerful and adverse prognostic factor.[6,7] For this reason, panelists at the National Cancer Institute State-of-the-Science Conference on Preoperative Therapy in 2007 recommended that "the preferred definition of pCR is the absence of residual invasive cancer within both the breast and lymph nodes."[8] As shown in Table 1, neither study showed that adding bevacizumab to chemotherapy achieved a significant improvement in the rate of pCR if both the breast and the nodes were factored into the definition.

The real dilemma posed by these results is to understand how to translate gains in a neoadjuvant treatment model into a decision about the suitability of treatment as standard adjuvant therapy. There are many barriers to this approach: the questions of short-term versus long-term benefit; the unknown relationship between incremental improvement in pCR and subsequent benefit in DFS; the different ways that treatments are employed in neoadjuvant versus adjuvant trials; the uncertain effects in various subgroups defined by grade, hormone-receptor status, HER2 status or molecular features; the impact of effective adjuvant therapies, particularly endocrine therapies; and the late adverse effects of therapy. It has been recently argued that the role for neoadjuvant trials in breast cancer is to make sure oncologists "don't pick the loser;"[9] that is, to use the neoadjuvant model to winnow out the ineffective agents before committing the intellectual, clinical and financial resources required of large, adjuvant trials. But nothing is easy for bevacizumab and breast cancer. Despite a collective experience with over 3,000 patients—hardly a small screening effort—it is unclear how to interpret the neoadjuvant results from NSABP B-40 and GeparQuinto when it comes to bevacizumab. Given the narrow difference in rates of pCR, the bearable but real increase in adverse effects with treatment, and the inconclusive data on how bevacizumab affects the natural history of advanced-stage breast cancer, the neoadjuvant data are insufficient for making bevacizumab a standard of current care. Only time, for maturation of long-term results from these two trials and other related studies analyzing the adjuvant role of bevacizumab, will settle the issue.

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Competing interests
The author declares no competing interests.

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GASTROINTESTINAL CANCER

Adjuvant chemotherapy after D2 gastrectomy for gastric cancer

Takaki Yoshikawa and Mitsuru Sasako

In the CLASSIC study, capecitabine–oxaliplatin was an effective chemotherapy after D2 gastrectomy for stage II–IIIB gastric cancer. We compared these data with the ACTS-GC study, which was the only pivotal study proving the benefit of adjuvant chemotherapy in these patients. Long-term survival data from CLASSIC are awaited with interest.

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Surgery in combination with adjuvant treatment is the globally accepted standard of care for stage II and III resectable gastric cancer. However, there are three different approaches to adjuvant treatment: in the USA, surgery followed by chemoradiotherapy is the standard protocol based on results from the INT-0116 trial;[1] in the UK

and some European countries, preoperative and postoperative chemotherapy with epirubicin, cisplatin, and 5-fluorouracil is used based on evidence from the MAGIC trial;[2] and in Japan, standard adjuvant treatment is single-agent postoperative chemotherapy with the oral fluoropyrimidine S-1 after D2 surgery based on results of the ACTS-GC trial.[3] These different approaches produced different survival results, which could not be explained by the difference of tumor biology based on varying ethnicity. Indeed, during the past decade, studies have elucidated the benefits of D2 gastrectomy and surgical undertreatment negatively affected the survival results of adjuvant treatment.[4,5] Thus, D2 gastrectomy is now the globally accepted surgical standard.[6,7] However, the optimal adjuvant therapy to use with D2 surgery has not been established.

Now, a study published in *The Lancet* (CLASSIC trial) by Bang *et al.*[8] reports that adjuvant chemotherapy with capecitabine and oxaliplatin after D2 surgery for patients from Asia with stage II or III gastric cancer resulted in significantly improved disease-free survival (DFS) compared with D2 surgery alone ($P<0.0001$). This trial is the first positive phase III study to show that doublet combination chemotherapy that included a platinum-based compound after D2 surgery is effective for gastric cancer.

> **"...evidence supports two approaches to adjuvant chemotherapy after D2 gastrectomy..."**

This randomized phase III study was well designed. The primary end point was DFS, a surrogate end point for overall survival. A total of 1,035 patients were enrolled for 37 months in 37 centers in South Korea (nearly 90% of the patients), China, and Taiwan. The study data were made available at the planned interim analysis time point with a median follow-up period of 2.9 years. The patient populations in the two arms were well balanced. Less than half of the patients had T3 tumors (T4 in Union for International Cancer Control TNM seventh edition), whereas 90% had nodal metastases. The tumors were located mostly in the gastric antrum and body (more than 80%), suggesting classic gastric cancer, and less than 3% of the participants had gastroesophageal junction cancer.

Toxic effects related to capecitabine and oxaliplatin were considered to be acceptable.[8] Major grade 3 or 4 toxic effects included neutropenia (22%), thrombocytopenia (8%), and nausea (8%). The therapy completion rate in the chemotherapy arm was 67%, which is quite high for gastric adjuvant chemotherapy that included a platinum-based compound; this high compliance likely contributed to the positive outcomes.

Table 1 | Comparisons between CLASSIC[8] and ACTS-GC[3]

Comparator	CLASSIC	ACTS-GC
Number of patients	1,035	1,059
Median age	56 years	63 years
Accrual period	37 months	39 months
Median follow-up period	2.9 years	2.9 years
Primary end point	DFS	OS
Tumor stage	T2 (54%), T3 (44%), node positive (90%)	T2 (54%), T3 (43%), node positive (89%)
Completion of chemotherapy	67% (6 months)	65.8% (12 months)
3-year DFS or RFS* with vs without chemotherapy (HR)	74% vs 59% (0.56 [95% CI 0.44–0.72])	72.2% vs 59.6% (0.62 [95% CI 0.50–0.77])
3-year OS with vs without chemotherapy (HR)	83% vs 78% (0.72 [95% CI 0.52–1.00])	80.1% vs 70.1% (0.68 [95% CI 0.52–0.87])

*RFS for ACTS-GC. Abbreviations: DFS, disease-free survival; OS, overall survival; RFS, recurrence-free survival.

Over the course of the study, the DFS curves clearly separated between the two arms.[8] In the chemotherapy group, 3-year DFS was 74%, which compared with 59% for the surgery-alone arm. The overall hazard ratio was 0.56. The hazard ratio was relatively constant in stage II (0.55), IIIA (0.57), and IIIB (0.57) disease, suggesting that capecitabine and oxaliplatin therapy was effective regardless of tumor stage. However, overall survival curves did not markedly separate between the two arms. The overall survival rate at 3 years was 83% in the chemotherapy arm and 78% in the surgery-alone arm; this similarity may have resulted from a number of factors. First, follow up for assessing overall survival as an end point has not been completed; nearly half (48%) of patients with recurrent disease in the surgery-alone arm were still alive at the time of the analysis. Second, survival after recurrence may differ between the two arms owing to the different rescue regimens available to the patients. Four types of cytotoxic drugs are used for the treatment of gastric cancer: fluoropyrimidine, platinum-based compounds, taxanes, and CPT-11. In the capecitabine and oxaliplatin arm, the patients had already been exposed to two of these key drugs during the adjuvant therapy; therefore, there were only two classes of drug available for chemotherapy after recurrence. By contrast, patients in the surgery-alone arm could receive all four drugs after recurrence. This difference may have affected survival after recurrence, which could shift the overall survival curve in favor of the surgery-alone arm. Third, DFS may be associated with event bias. Although imaging was performed every 6 months during the first 3 years and every year thereafter, none of the presented data show that the intervals between imaging were consistent in the two arms. DFS could be shortened in the control arm if the patients were checked earlier—for example, in response to small increases of tumor marker levels or subtle clinical signs suggestive of recurrence.

There are several differences between the two trials (ACTS-GC and CLASSIC) that have assessed adjuvant chemotherapy in this patient population (Table 1).[3] The two trials were similar in a large number of areas: the number of patients, accrual period, median follow-up period at the time the analysis was performed, surgery, and tumor and nodal stage; however, the median patient age was 7 years younger in the CLASSIC than in the ACTS-GC trial. Toxic effects were mostly mild, although the duration of chemotherapy was longer in the ACTS-GC study. DFS curves from the CLASSIC study and recurrence-free survival (RFS) curves from the ACTS-GC study clearly separated between the chemotherapy and the surgery-alone arms; hazard ratios for DFS (CLASSIC) or RFS (ACTS-GC) were similar and relatively low. Interestingly, the hazard ratios were similar regardless of tumor stage in the CLASSIC study, whereas the hazard ratio increased for patients with more-advanced stage disease in the ACTS-GC trial. The primary difference was that the overall survival curves clearly separated in the

Practice point

In patients treated with D2 surgery for stage II or III gastric cancer, capecitabine and oxaliplatin without radiotherapy is an appropriate option.

ACTS-GC data but not in the CLASSIC data despite a similar median follow-up period.

Although D2 gastrectomy is globally accepted among surgeons as the surgical standard of care for curable gastric cancer, some medical oncologists claim that the difference in the overall survival between the Asian[3,8] and INT-0116[1] studies could be attributable to putative East–West differences in tumor biology that have yet to be documented for gastric cancer. In the editorial regarding the published data of the 5-year results of ACTS-GC,[8] Macdonald[9] completely ignored the significant and well-documented survival impact of surgical undertreatment. Indeed, recently reported results of a Korean study on postoperative chemoradiotherapy after D2 gastrectomy did not show a benefit from chemoradiotherapy over postoperative chemotherapy alone,[10] which confirmed that radiation simply compensated for the effect of D2 surgery in patients who underwent surgical undertreatment.

In conclusion, evidence supports two approaches to adjuvant chemotherapy after D2 gastrectomy for resectable gastric cancer: S-1 for 1 year, and capecitabine and oxaliplatin for 6 months. With capecitabine and oxaliplatin, the chemotherapy period is shorter but the treatment is more toxic compared with S-1. Balancing the risks and efficacy of treatment, S-1 seems to be appropriate for patients with stage II disease and capecitabine and oxaliplatin seems attractive for patients with stage III resectable gastric cancer. Direct comparison of these approaches should be undertaken after the long-term data from the CLASSIC trial are available.

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Competing interests

M. Sasako declares an association with the following companies: Chugai Pharmaceuticals, Sanofi-Aventis, Taiho Pharmaceuticals. See the article online for full details of the relationships. T. Yoshikawa declares no competing interests.

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GYNECOLOGICAL CANCER

First-line bevacizumab for ovarian cancer—new standard of care?

Susana Banerjee and Stan B. Kaye

Demonstration of the clinically significant activity of bevacizumab in advanced-stage ovarian cancer has attracted a great deal of interest. Here, we summarize the two positive phase III trials that led to EMA approval of bevacizumab as first-line therapy and discuss the optimum use of the drug in this disease.

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In December 2011, two positive phase III trials[1,2] that assessed bevacizumab in patients with ovarian cancer were reported in the *New England Journal of Medicine*; these results led to the EMA approval of the drug as first-line treatment in combination with carboplatin and paclitaxel for this disease.[3] Bevacizumab is currently the most widely tested antiangiogenic agent for the treatment of cancer. Bevacizumab is a monoclonal antibody that targets the VEGF pathway, which has a critical role in ovarian function as well as in the spread of ovarian cancer.[4] Therefore, positive results from clinical trials assessing bevacizumab in this notoriously difficult-to-treat disease have been eagerly anticipated.

The first study (GOG-0218) was reported by Burger *et al.*[1] and was a double-blind, three-arm, placebo-controlled study in 1,873 patients with newly diagnosed stage III (incompletely resected with residual disease >1 cm) or stage IV epithelial ovarian cancer. Patients were randomly assigned to one of three treatments: combination chemotherapy (carboplatin–paclitaxel), carboplatin–paclitaxel chemotherapy plus concurrent bevacizumab or carboplatin–paclitaxel chemotherapy plus concurrent and maintenance bevacizumab. The bevacizumab dose was 15 mg/kg for up to 22 cycles (15 months total). After a protocol amendment, stage III patients with macroscopic residual disease of ≤1 cm were also included. Nevertheless, all patients enrolled had advanced-stage disease and their overall outlook was worse than those patients assessed in the second study, ICON7.[2]

Perren *et al.*[2] published the results from the ICON7 study. The trial randomly assigned patients to one of two arms: 1,528 patients received carboplatin–paclitaxel chemotherapy with or without concurrent and maintenance bevacizumab. Bevacizumab was given at 7.5 mg/kg (half the dose used in GOG-0218) for a total of 18 cycles (12 months total). In this trial, 9% of patients had high-risk, early stage disease (FIGO stage I or IIA, clear cell or grade 3 histology) whereas 30% were at the

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ORIGINAL ARTICLE

Decreased FANCJ caused by 5FU contributes to the increased sensitivity to oxaliplatin in gastric cancer cells

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Abstract
Background Oxaliplatin is effective against many types of cancer, and the combination of 5-fluorouracil (5FU) and oxaliplatin is synergistically effective against gastric cancer, as well as colon cancer. The FANCJ protein is one of the Fanconi anemia (FA) gene products, and its interaction with the tumor suppressor BRCA1 is required for DNA double-strand break (DSB) repair. FANCJ also functions in interstrand crosslinks (ICLs) repair by linking to mismatch repair protein complex MLH1-PMS2 (MutLα). While oxaliplatin causes ICLs, 5FU is considered to cause DSBs. Therefore, we investigated the importance of FANCJ in the synergistic effects of oxaliplatin and 5FU in MKN45 gastric cancer cells and the derived 5FU-resistant cell line, MKN45/F2R.
Methods MKN1, TMK1, MKN45, and MKN45/F2R (5FU-resistant) gastric cancer cells were treated with 5FU and/or oxaliplatin. The signaling pathway was evaluated by a western blotting analysis and reverse transcription polymerase chain reaction (RT-PCR). Drug resistance was evaluated by the 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay.
Results In MKN45 cells, the combination of 5FU and oxaliplatin had synergistic effects. DSBs appeared when the cells were treated with 5FU. FANCJ was down-regulated, and BRCA1 was induced in a dose- and time-dependent manner. MKN45 cells showed increased sensitivity to oxaliplatin when FANCJ was knocked down by short interfering (si) RNA. However, these findings were not observed in MKN45/F2R 5FU-resistant cells.
Conclusion These results strongly suggest that the decrease in FANCJ caused by 5FU treatment leads to an increase in sensitivity to oxaliplatin, thus indicating that the FANCJ protein plays an important role in the synergism of the combination of 5FU and oxaliplatin.

Keywords Fluorouracil · Oxaliplatin · BACH1 protein

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Introduction

Gastric cancer remains one of the major causes of cancer deaths around the world [1, 2]. Most patients with advanced and metastatic gastric cancer are treated with chemotherapy, and the combination of S-1 and cisplatin (CDDP) is one of the standard first-line regimens used in Japan [3].

The combination of fluorouracil (5FU) and oxaliplatin is used in the fluorouracil, leucovorin, and oxaliplatin (FOLFOX) regimen for colorectal cancer, and its efficacy has been clinically confirmed [4]. Oxaliplatin exerts growth inhibitory effects on many cancer cell lines and tumors, including some that are primarily resistant to CDDP and carboplatin. This increased activity is due to its 1, 2-diaminocyclohexane (DACH) carrier ligand, which provides higher lipophilicity, as evidenced by its large volume of distribution and slow excretion through the kidneys [5]. The combination of 5FU and oxaliplatin against gastric cancer

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has been demonstrated to be effective in the clinic [6, 7], and oxaliplatin is sometimes used to replace CDDP for the treatment of gastric cancer, because of its better tolerability [8]. Oxaliplatin and 5FU have demonstrated activity against colon cancer cell lines, and synergistic activity between the agents has been observed in experimental models [9, 10], but the mechanism underlying their synergistic effect is unclear.

The FANCJ protein is one of the Fanconi anemia (FA) gene products. It was first identified as a protein that binds directly to the breast cancer-associated tumor suppressor, BRCA1 [11, 12], and was originally named BACH1/BRIP1 [12, 13]. Fanconi anemia is a rare hereditary disorder characterized by skeletal abnormalities, bone marrow failure, and an increased incidence of cancer. The basic cellular abnormality in FA has been postulated to lie in the DNA repair mechanisms, because cells from FA patients display chromosomal abnormalities and are hypersensitive to agents that cause DNA interstrand crosslinks (ICLs), such as mitomycin C (MMC) and CDDP [14]. The role of FANCJ in the FA pathway has not yet been completely elucidated. So far, it has been shown that FANCJ is a DNA helicase for the D-loop structure in the early stage of the homologous recombination (HR) pathway of double-strand break (DSB) repair; therefore, the association of FANCJ with BRCA1 is essential for DSB repair [12, 13]. Moreover, FANCJ interacts with the mismatch repair complex MutLα, composed of MLH1 and PMS2, independent of BRCA1, and the FANCJ/MutLα interaction is essential for ICL repair [15].

It is known that 5FU induces DSBs as a result of its incorporation into DNA [16] or thimidylate synthase (TS) inhibition [17], and oxaliplatin induces ICLs by its pharmacological action. Based on these facts, we hypothesized that the two functions of FANCJ would be involved in the synergistic effects of 5FU and oxaliplatin against gastric cancer.

In the present study, we clarified the differential regulation of the FANCJ protein between 5FU-sensitive and 5FU-resistant cells and also demonstrated the mechanism underlying the synergistic effects of 5FU and oxaliplatin against gastric cancer cells.

Materials and methods

Drugs

5FU was purchased from Kyowa Hakko (Tokyo, Japan), and oxaliplatin was purchased from Yakult Honsha (Tokyo, Japan).

Cell lines and cell culture

Gastric cancer cell lines (MKN45, MKN1, TMK1) were cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), antibiotics (Sigma-Aldrich), and HEPES (Sigma-Aldrich) in a humidified atmosphere of 5 % CO_2 at 37 °C. MKN45 and TMK1 are poorly differentiated human gastric adenocarcinoma cell lines. MKN1 is an adenosquamous carcinoma cell line. MKN45/F2R is a 5FU-resistant cell line. To establish this cell line, the MKN45 parent cells were continuously exposed to increasing concentrations (0.1–2 μM) of 5FU over a period of 1 year. The MKN45/F2R cells were routinely maintained in culture medium containing 2 μM of 5FU. To eliminate the effects of 5FU in our experiments, the resistant cells were cultured in a drug-free medium for at least 2 weeks before all of the studies [18].

3-(4,5-Dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay for the effects of 5FU or oxaliplatin on cell viability

Cell growth was assessed with a standard MTT assay, which detects the dehydrogenase activity in viable cells. A total of 5×10^3 cells were seeded in each well of 96-well culture plates. After 24 h, the cells were treated with various concentrations of drugs. After another 72 h, the culture medium was removed, and 100 μl of a 0.5 mg/ml solution of MTT (Sigma-Aldrich) was added to each well. The plates were then incubated for 4 h at 37 °C. The MTT solution was then removed and replaced with 100 μl of dimethyl sulfoxide (Wako) per well, and the absorbance at 540 nm was measured using an Envision 2104 Multilabel Reader (Perkin Elmer, Waltham, MA, USA).

The Combination Index (CI) was calculated by the formula $CI = A/Ax + B/Bx$ (A: the 50% inhibitory concentration [IC50] for drug A in combination, Ax: the IC50 for drug A alone, B: the IC50 for drug B in combination, Bx: the IC50 for drug B alone) (based on the Loewe additivity model [19]).

Immunofluorescence for γH2AX

The cells were harvested in a Lab-Tek Chamber Slide System (Thermo Fisher Scientific, Waltham, MA, USA) and immunofluorescence studies were performed. The cells were first fixed in 4 % paraformaldehyde for 15 min at room temperature and washed three times with phosphate-buffered saline (PBS) containing 1 % Triton X-100 (PBST). Blocking against non-specific binding was performed for 60 min with 0.5 % goat serum dissolved in PBST, and the cells were again washed three times with PBST. The rabbit monoclonal anti-phospho-H2AX antibody (Cell Signaling Technology, Danvers, MA, USA, 1:200) was used as the primary antibody. The cells were incubated for 1 h at room temperature with the primary antibody dissolved in PBST

supplemented with 0.5 % goat serum, and then the cells were washed three more times with PBST. The cells were then incubated with highly cross-adsorbed Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA, 4 μg/ml), Phalloidin Alexa Fluor 488 Conjugate (Lonza, Walkersville, MD, USA, 1:40), and 4', 6-diamidino-2-phenylindole (DAPI) Nucleic Acid Stain (Invitrogen 1:25000) in PBST containing 0.5 % goat serum. Images were acquired on a DP70-WPC02 camera mounted on an IX50 system (Olympus, Tokyo, Japan).

Immunoprecipitation, western blot analysis, and antibodies

Cells were harvested and lysed in CelLytic™ M (Sigma-Aldrich) for 30 min on ice. The protein concentration of the lysates was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). For the immunoprecipitation assays, cell lysates were incubated with an anti-FANCJ antibody (Abcam, Cambridge, UK, 1:100) for 2 h at 4 °C and PureProteome™ Protein A Magnetic Beads (Millipore, Billerica, MA, USA) were added, and the beads were subsequently washed. The cell lysates were boiled in Sample Buffer Solution (Wako), then total cell protein extracts (20 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using SuperSep™ (Wako), and they were electrophoretically transferred onto polyvinyl difluoride (PVDF) membranes. The membranes were blocked with PVDF blocking reagent (TOYOBO, Osaka, Japan) for 1 h. The membranes were then incubated with primary antibodies against β-actin, FANCJ, BRCA1, FANCD1/BRCA2, phospho-Histone H2AX(Ser139) (Cell Signaling Technology, 1:5000), MLH1 (Abcam, 1:100000), FANCD2 (Abcam, 1:50000), and PMS2 (EPITOMICS, San Francisco, CA, USA, 1:20000) overnight at 4 °C. The primary antibodies were diluted with Can Get Signal Solution 1 (TOYOBO). The membranes were then washed with Dako Washing Buffer (Dako, Glostrup, Denmark) and incubated with the appropriate secondary antibodies (Millipore, 1:25000). Secondary antibodies were diluted with Can Get Signal Solution 2 (TOYOBO). The immunoreactive proteins were visualized by chemiluminescence using ImmunoStar LD reagents (Wako), and images were captured by an LAS-4000 system (FUJIFILM, Tokyo, Japan).

Transfection and small interfering RNA experiments for FANCJ

The MKN45 cells were cultured in medium without antibiotics for 24 h before transfection at 50–70 % confluence. The cells were transfected with a small interfering RNA (siRNA) oligonucleotide using Lipofectamine RNAiMAX (Invitrogen) in a final siRNA concentration of 40 nmol/l in serum-free Opti-MEM (Invitrogen). After 48 h, the total RNA and proteins were extracted, and the expression levels of the FANCJ mRNA and protein were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) and a western blotting analysis, respectively. The siRNA oligonucleotides (Stealth RNAi) and the negative control oligonucleotides (Stealth RNAi siRNA Negative Control) for FANCJ were purchased from Invitrogen.

Results

The combination of 5FU and oxaliplatin has synergistic effects against MKN45 cells

To verify that there were synergistic effects of 5FU and oxaliplatin against gastric cancer cells, we performed the MTT assay using 5FU and oxaliplatin in MKN1, TMK1, MKN45, and MKN45/F2R (5FU-resistant) cells (Fig. 1a–d), and calculated the IC50 and the CI using the Loewe additivity model [19] (Table 1). The MKN45/F2R cells were previously established as 5FU-resistant cells in our laboratory [18]. The IC50 of MKN45/F2R cells for 5FU in the present study was 52.4 μM, which is 46.0-fold increased resistance compared with the parent MKN45 cell line, for which the IC50 of 5FU was 1.14 μM, while the major characteristics of these cell lines were consistent, as reported previously [18]. In the MKN45 cells, when 0.1 μM of 5FU was combined with oxaliplatin, the CI was 0.439, which was significantly lower than 1 ($p < 0.05$). This means that the combination had a synergistic effect. Conversely, no synergistic effect was observed in the MKN1, TMK1, and MKN45/F2R cells.

Changes in ICL repair proteins after 5FU treatment

Oxaliplatin induces its cytotoxic effects primarily by inducing ICLs. We herein examined the differential expression of the proteins involved in ICL repair by a western blotting analysis after treating MKN45 gastric cancer cells with 1 μM, 10 μM, or 100 M of 5FU for 24 h. The proteins examined included FANCJ, BRCA1, MLH1, PMS2, FANCD2, and FANCD1/BRCA2. The FANCJ protein, which is one of the FA gene products, and the tumor suppressor BRCA1 are required to repair DSBs [12, 13]. FANCJ also functions in ICL repair by linking to mismatch repair protein complex MLH1-PMS2 (MutLα) [15]. FANCD1/BRCA2 and FANCD2 are the key proteins in the FA pathway [14]. Interestingly, we observed that the expression of the FANCJ protein was decreased in a dose-dependent manner, and the expression was decreased to 48 % at 100 μM of 5FU compared to the expression level without 5FU. On the other hand, the expression of the

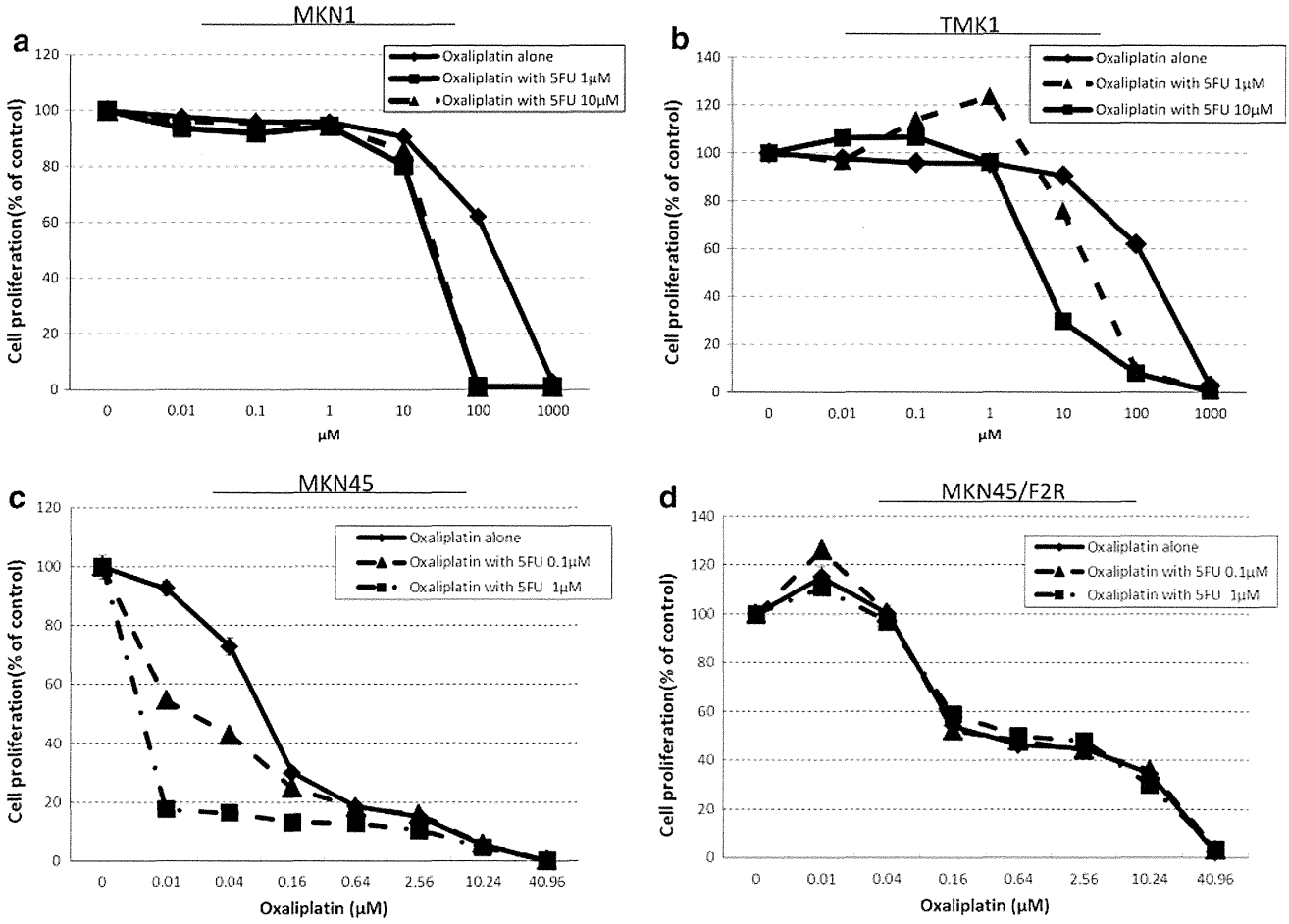

Fig. 1 The in vitro sensitivity of the MKN1, TMK1, MKN45 and MKN45/F2R cells to oxaliplatin and/or 5-fluorouracil (*5FU*). **a**, **b**, **d** No synergistic effect was observed at any concentration of 5FU in the MKN1, TMK1, and MKN45/F2R cells. **c** In the MKN45 cells, when 5FU was combined with oxaliplatin, a synergistic effect was observed

Table 1 IC50 values for 5FU and/or oxaliplatin in gastric cancer cells

Drug	MKN1	TMKN1	MKN45	MKN45-F2R
5FU alone	205.50 ± 4.62	297.89 ± 8.92	1.14 ± 0.888	52.4 ± 8.35
Oxaliplatin alone	159.65 ± 4.21	400.66 ± 8.32	0.177 ± 0.00992	2.58 ± 0.311
Oxaliplatin with 0.1 μM 5FU	24.116 ± 0.3425	25.539 ± 1.6378	0.0877 ± 0.00126*	0.317 ± 0.474
Oxaliplatin with 1 μM 5FU	26.315 ± 0.5236	4.99 ± 0.4615	–	0.61 ± 0.526

The 50% inhibitory concentration (IC50) values were calculated from the results of the MTT assay for oxaliplatin and/or 5-fluorouracil (5FU) in the MKN1, TMK1, MKN45, and MKN45/F2R cells. The combination index (CI) was calculated using the Loewe additivity model [19], and a synergistic effect was observed when 0.1 μM of 5FU was combined with oxaliplatin in MKN45 cells (CI = 0.439 ± 0.077**). The IC50 value could not be calculated for these cells when 1 μM of 5FU was combined with oxaliplatin, because the IC50 value was lower than the lowest concentration used in this experiment

* $p < 0.05$ based on Student's *t*-test

** $p < 0.05$ based on Student's *t*-test compared to 1

BRCA1 protein was increased by 2.1-fold after treatment with 1 μM of 5FU. These changes indicated that FANCJ and BRCA1 functioned to repair the DSBs caused by 5FU, and these proteins were likely to be related to the synergism between 5FU and oxaliplatin, because a deficit of FANCJ protein leads to a failure of ICL repair [15]. None of the expression levels of other proteins involved in DSB or ICL repair, such as MutLα, were changed, or they were only slightly increased after 5FU treatment, and seemed not to be involved in the synergism between 5FU and oxaliplatin.

We also examined the expressions of FANCJ and BRCA1 in other gastric cancer cell lines, such as MKN1,

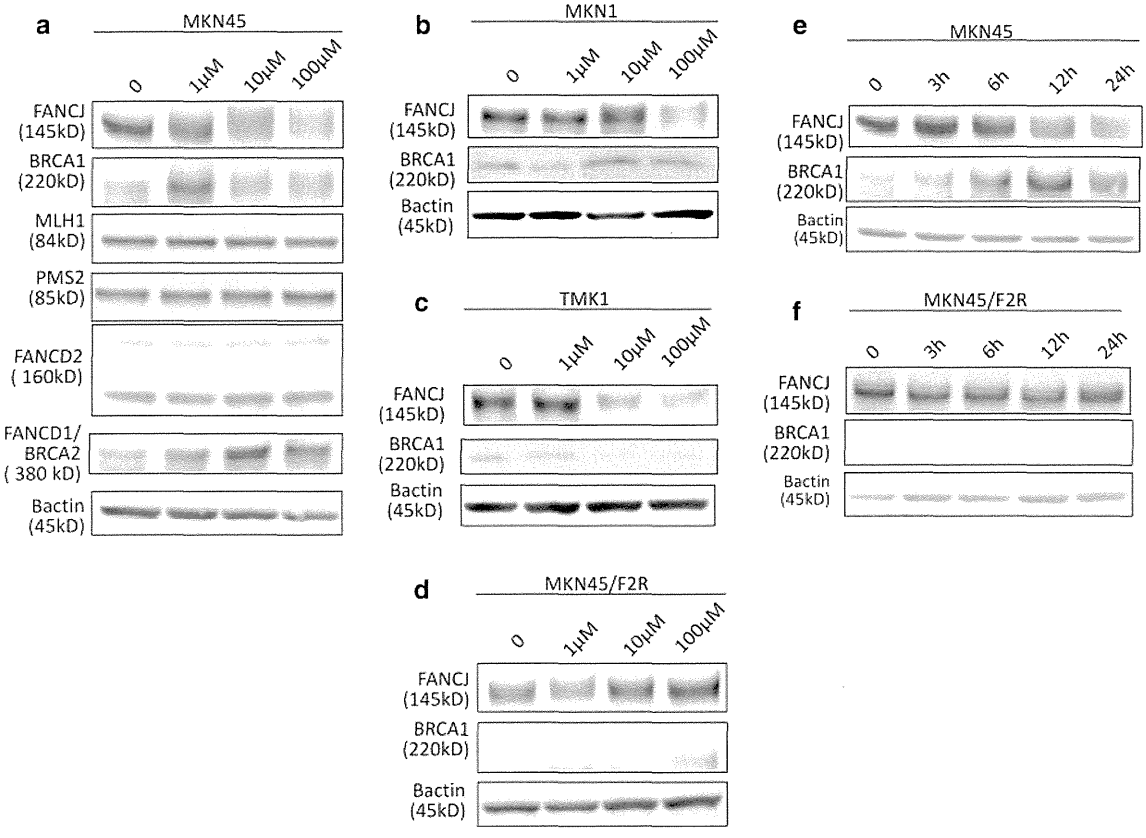

Fig. 2 Changes in interstrand crosslink (ICL) repair proteins after 5FU treatment. **a** The results of a western blotting analysis of the expression of FANCJ, BRCA1, MLH1, PMS2, FANCD2, and FANCD1/BRCA2 in MKN45 cells treated with 5FU at 1, 10, and 100 μM for 24 h. **b** The results of the western blotting analysis of FANCJ and BRCA1 in MKN1 cells. **c** The results of the western blotting analysis in TMK1 cells. **d** The results of the western blotting analysis in MKN45/F2R cells. **e** The results of the western blotting analysis of the expression of FANCJ and BRCA1 in MKN45 cells treated with 10 μM of 5FU for 3, 6, 12, and 24 h. **f** The results of the western blotting analysis of the expression of these proteins in MKN45/F2R cells treated with 10 μM of 5FU for 3, 6, 12, and 24 h

TMK1, and MKN45/F2R cells. As shown in Fig. 2b–d. The downregulation of FANCJ was reproduced in MKN1 and TMK1 cells, and induction of BRCA1 was also observed in MKN1 cells. In the MKN45/F2R cells, both FANCJ and BRCA1 were unchanged after 5FU treatment.

We then treated MKN45 and MKN45/F2R cells with 10 μM of 5 FU for 3, 6, 12, and 24 h and examined the FANCJ and BRCA1 expression levels by a western blot analysis; as shown in Fig. 2e, f the FANCJ expression in the MKN45 parental cells was decreased and BRCA1 expression was increased in a time-dependent manner. The FANCJ protein was decreased to 48 % of the level of the control after a 24-h treatment, while the expression of BRCA1 was increased by 4.3-fold compared to the control level. These changes were not observed in MKN45/F2R cells.

DSBs appeared when MKN45 cells were treated with 5FU

It has previously been established that 5FU induces DSBs, and FANCJ functions in DSB repair [12, 13]. Therefore, we examined whether DSBs occurred in MKN45 and MKN45/F2R cells treated with 5FU.

To evaluate the DSB status, we performed immunofluorescence studies for γH2AX, which is a marker of DSBs [20, 21]. There were indeed DSBs, which are indicated in red in Fig. 3a. The MKN45 and MKN45/F2R cells were treated with 5FU at concentrations of 1, 10, and 100 μM for 24 h, and we found that DSBs were increased in a dose-dependent manner in the MKN45 parental cells, while this phenomenon was not observed in MKN45/F2R cells (Fig. 3a). We also treated the cells with 10 μM of 5FU for 3, 12, and 24 h, and examined the DSBs (Fig. 3b). As expected, the DSBs were observed in MKN45 parental cells, and they were increased in a time-dependent manner, with DSBs being present in 62 % of the cells after the 24-h treatment. However, no time-dependent DSBs were detected in the MKN45/F2R cells.

Next, we performed a Western blot analysis for γH2AX after 5FU treatment to confirm the increased expression of the protein. The expression of γH2AX was increased by 6.2-fold after treatment with 10 and 100 μM of 5FU for