TABLE VI. Lymph Node Metastasis of MDCT Staging and Pathological Diagnosis

| CT-N | pN0 | pN1 | pN2 | pN3 | pN1/2/3 | Total |
|------|-----|-----|-----|-----|---------|-------|
| CT-N0 | 23 | 5 | 7 | 6 | 18 | 41 |
| CT-N1 | 3 | 0 | 1 | 0 | 1 | 4 |
| CT-N2 | 5 | 1 | 5 | 4 | 10 | 15 |
| CT-N3 | 3 | 1 | 1 | 0 | 2 | 5 |
| CT-N1/2/3 | 11 | 2 | 7 | 4 | 13 | 24 |
| Total | 34 | 7 | 14 | 10 | 31 | 65 |

$P = 0.6631$.

of these patients was negative for arterial invasion, but showed a high positive rate of dissected margin. It should be noted that MDCT was performed with at least an interval of 0.625 mm, and the slides of the fixed specimen for pathological diagnosis should be at an interval of 5 mm for each. It is possible that pathological diagnosis underestimates arterial invasion because of the 5-mm interval. Furthermore, MDCT showed only intraluminal space of the vessels and it is difficult to discuss the relationship between the tumor and the vessel wall itself in CT. This methodological limitation may also impact the high positive rate of the dissected margin and this should lead to poor prognosis in CT-A 1/2/3 patients. Thus, fCur A/B (R0) resection and the touch smear cytology [14] might be useful for accurate diagnosis and decision on arterial resection.

The next question about arterial invasion is prognosis of patients with pathologically positive tumors for arterial invasion (pA (+)). In this study, only one patient was positive for pathological arterial invasion after fCur A/B (R0) resection, and thus we could not evaluate prognosis according to pathological arterial invasion.

On the other hand, the other factors of T category (CT-PV and CT-S), with the exception of CT-Hinf/Ginf/Binf/Panc, did not correlate with prognosis and curative resection. CT-Hinf/Ginf/Binf/Panc was identified as a prognostic factor in univariate analysis and showed marginal statistical significance in multivariate analysis, although it did not correlate with curative resection. Thus, this factor is a potentially suitable prognostic factor but not non-curative surgery factor, although further studies of larger population samples are needed to confirm these results.

For the N category, CT-N was neither a prognostic factor nor a non-curative surgical factor. Previous reports indicated that the detection of pathological metastasis to lymph node is difficult with a sensitivity of approximately 50% [6–10]. In our study, there was no relationship between MDCT-based diagnosis and pathological metastasis. The CT-criteria used for lymph node metastasis differed slightly among various groups; however, the sensitivity was low in each study. MDCT could not detect lymph node metastasis and therefore could not rule out lymph node metastasis-related non-curative (fCurC, R2) resection based on the diagnostic criteria. Another imaging modality, the F-fluorodeoxyglucose–positron emission tomography (FDG–PET) and/or magnetic resonance imaging (MRI), provides a better detection of pathological lymph node metastasis; however, the sensitivity of the diagnosis using FDG–PET is still low (~50%) under unclear cut-off information of standardized uptake values (SUV)max [15–17], and the diagnosis of lymph node metastasis in biliary cancer by MRI has rarely been reported. Based on this background, preoperative detection of lymph node metastasis in biliary cancer is limited even by the most advanced imaging modalities. To avoid fCur C (non-curative; R2) resection based on lymph node or other distant metastasis, we should develop new diagnostic criteria or modality; for example, methionine PET [18] or circulating tumor cells (CTC) [19–22]. Especially for CTC, we reported recently that CEA estimated by quantitative polymerase chain reaction (qPCR) is a good marker for the detection of micrometastasis [19]. In this regard, CTC is used in hepatocellular carcinoma to predict recurrence and poor prognosis [19–22]. Further studies are needed to determine whether circulating CEA-positive cells in biliary cancer could be used to predict regional and/or distant metastasis.

Using MDCT, our study detected preoperative prognostic factors and non-curative surgery factors, including arterial invasion. This factor relates to local extension but not to regional metastasis (e.g., regional lymph node metastasis). This result would be helpful in deciding the eligibility criteria for neoadjuvant therapy. Recent studies described new regimens of anti-cancer drugs for biliary cancer, including gemcitabine with cisplatin, oxaliplatin, and capecitabine [23–27]. Furthermore, recent clinical trials of chemoradiotherapy for unresectable locally advanced biliary cancer have been reported [28–30]. Perhaps, the next treatment strategy would include neoadjuvant chemoradiotherapy similar to that available for locally advanced pancreatic cancer with suspected arterial and/or portal

TABLE VII. MDCT-Evaluated Hepatic Artery Invasion and Pathological Invasion

| | pA0 | pA1 | pA2 | pA3 | pA1/2/3 | Total |
|------|-----|-----|-----|-----|---------|-------|
| fCurA/B | | | | | | |
| CT-A0 | 29 | 0 | 0 | 0 | 0 | 29 |
| CT-A1 | 10 | 0 | 0 | 1 | 1 | 11 |
| CT-A2 | 0 | 0 | 0 | 0 | 0 | 0 |
| CT-A3 | 1[a] | 0 | 0 | 0 | 0 | 1 |
| CT-A1/2/3 | 11 | 0 | 0 | 1 | 1 | 12 |
| Total | 40 | 0 | 0 | 1 | 1 | 41 |
| fCurC | | | | | | |
| CT-A0 | 3 | 0 | 0 | 0 | 0 | 3 |
| CT-A1 | 8 | 0 | 2 | 0 | 2 | 10 |
| CT-A2 | 1[a] | 0 | 0 | 0 | 0 | 1 |
| CT-A3 | 2[a] | 0 | 0 | 0 | 0 | 2 |
| CT-A1/2/3 | 11 | 0 | 2 | 0 | 2 | 13 |
| Total | 14 | 0 | 2 | 0 | 2 | 16 |

[a]Ipsilateral hepatic artery of the resected liver.

TABLE VIII. MDCT-Evaluated Hepatic Artery Invasion in Hilar Biliary Cancer

| CT-A | n | Contralateral hepatic artery resection | pA0 | pA123 |
|---|---|---|---|---|
| Total | 23 | 2 | 18 | 1 |
| fCurA/B | 12 | 2 | 10 | 1 |
| CT-A0 | 1 | 1[a] | 1 | 0 |
| CT-A1 | | | | |
| Ipsilateral to resected liver | 9 | 1[a] | 8 | 1[b] |
| Contralateral to resected liver | 1 | 0 | ND | ND |
| CT-A2/3 | | | | |
| Ipsilateral to resected liver | 1 | 0 | 1 | 0 |
| Contralateral to resected liver | 0 | 0 | 0 | 0 |
| fCurC | 11 | 0 | 8 | 1 |
| CT-A0 | 1 | 0 | 1 | 0 |
| CT-A1 | | | | |
| Ipsilateral to resected liver | 5 | 0 | 4 | 1[b] |
| Contralateral to resected liver | 2 | 0 | ND | ND |
| CT-A2/3 | | | | |
| Ipsilateral to resected liver | 3 | 0 | 3 | 0 |
| Contralateral to resected liver | 0 | 0 | 0 | 0 |

ND, not determined.
[a]pA0 at the resected contralateral hepatic artery.
[b]At ipsilateral hepatic artery of the resected liver.

invasion [31,32]. In biliary cancer, portal invasion is not a prognostic factor [33], and suspected arterial invasion is indicative of poor prognosis and a criterion for neoadjuvant chemoradiation. Thus, our findings should have an impact on the diagnosis-related decision-making regarding treatment strategy.

In this study, we evaluated just before surgery and after percutaneous transhepatic portal vein embolization (PTPE) in the patients who required this procedure (n = 11). The latter was provided to improve liver regeneration, which impacts the rate of curative (fA/B, R0) resection and prognosis of patients. For actual analysis of intention-to-treat at the first contact and after the decision for surgery, we should include information obtained before PTPE.

In conclusion, the present study demonstrated that suspected arterial invasion, as detected by MDCT, could predict poor prognosis of patients with biliary cancer after surgery. Suspected arterial invasion was a non-curative surgical factor associated with positive surgical margin. This MDCT-based preoperative factor could be useful for clinical decision-making regarding neoadjuvant therapy in combination with surgery.

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# TDGF1 is a novel predictive marker for metachronous metastasis of colorectal cancer

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**Abstract.** Teratocarcinoma-derived growth factor 1 (*TDGF1*) is a member of the epidermal growth factor-cripto *FRL1* cryptic protein family and is involved in the activation of several different signaling pathways during embryonic development and cellular transformation. Previous reports show that *TDGF1* regulates the activation of several signaling pathways and controls cellular transformation in embryonic status, whereas its significance in colorectal cancer (CRC) is not yet fully understood. The present study comprised 55 patients who underwent surgery for CRC, as well as two cell lines derived from human CRC. The correlation of gene expression with clinical parameters in patients was assessed. The biological significance of *TDGF1* expression was evaluated by knock-down experiments in the cell lines. Seventeen of 55 (30.9%) cases exhibited a higher *TDGF1* expression in cancerous regions than in marginal non-cancerous regions. Patients with high *TDGF1* expression were statistically susceptible to a recurrence of the disease, and showed poorer disease-free survival than those with low expression. The assessment of *TDGF1* knock-down in the 2 cell lines demonstrated that the siRNA inhibition resulted in a statistically significant reduction in cell growth and invasion. In conclusion, the present data strongly suggest the usefulness of *TDGF1* as a predictive marker for metachronous metastasis in CRC patients.

## Introduction

Cancer is a prominent malignancy in many developed countries, including the United States and Japan (1,2). The incidence of colorectal cancer (CRC) has increased significantly in recent years in concert with the changing lifestyle (3). The major cause of death from CRC is liver

metastases (4). Although treatment has recently improved, it fails in approximately one-third of patients, who require an alternative strategy (2). Thus, useful predictive markers are needed for CRC patients.

Tumor-promoting oncogenes and tumor suppressors control cell proliferation through CRC cell cycle arrest (1,5,6). Identifying additional genes responsible for the development and progression of CRC, as well as understanding their clinical significance would improve diagnosis and treatment of disease. The characterization of key molecules is particularly promising for the development of novel approaches to treat gastrointestinal tumors.

The human teratocarcinoma-derived growth factor 1 (*TDGF1*) gene is a member of the epidermal growth factor-cripto *FRL1* cryptic gene family and was initially isolated from human teratocarcinoma (7). *TDGF1* is expressed in several types of human tumors and has been detected by immuno-histochemistry in the breast, stomach, colon, pancreas, and lung (8-16). For gastric cancer, the combined expression of *TDGF1* and E-cadherin is reported as a prognostic factor (16).

We investigated the importance of the *TDGF1* gene by analyzing it in 55 consecutive paired cases of CRC and non-cancerous regions as well as in 2 CRC cell lines. We propose that *TDGF1* expression is important for prognostic evaluation and suggest that *TDGF1* could be a novel marker for CRC prognosis.

## Materials and methods

*Clinical tissue samples.* The study comprised 55 consecutive patients who underwent surgery for CRC at Osaka University from 2003 to 2004. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written, informed consent was confirmed in accordance with the institutional ethics guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin, and sectioned with hematoxylin and eosin. All specimens were frozen immediately in liquid nitrogen after resection and kept at -80°C until RNA extractions. After surgery, patients were followed up with a blood examination including the tumor markers serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9), as well as imaging modalities, such as abdominal ultrasonography, computed

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tomography, and chest X-ray every 3-6 months. Clinico-pathological factors were assessed according to the tumor-node-metastasis (TNM) criteria classification of the International Union Against Cancer (UICC) (17).

*Cell lines and culture.* Two cell lines derived from human CRC, HCT116 and LoVo, were used in this study (18,19). They were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified $CO_2$ atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human *TDGF1*, (5'-AAGACUUUAGAAAUGGCCAUGAUCC-3'/5'-GGAUCAUGGCCAUUUCUAAAGUCUU-3', 5'-UUUA CUGGUCAUGAAAUUUGCAUGA-3'/5'-UCAUGCAAAU UUCAUGACCAGUAAA-3', and 5'-UGGACGAGCAAAU UCCUGAUGGCCC-3'/5'-GGGCCAUCAGGAAUUUGCU CGUCCA-3'), as well as negative control siRNA (NC) were purchased in the Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA). CRC cell lines were transfected with siRNA at a concentration of 20 $\mu$mol/l using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen), treated in accordance with the manufacturer's protocols, and analyzed by proliferation assay. All siRNA duplexes were used together as a triple transfection. The number of cell cultures was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in the two CRC cell lines to evaluate proliferation and invasion under *TDGF1* suppression. Each cell line with siRNA was compared to the wild-type and a negative control. Values were expressed as the mean ± standard error of mean (SEM) from five independent experiments.

*RNA preparation and quantitative real-time reverse transcrip-tase-polymerase chain reaction (RT-PCR).* Total RNA was prepared using TRIzol reagent and a PureLink RNA Mini kit (Invitrogen) in accordance with the manufacturer's protocols. RNA was reverse transcribed with SuperScriptIII (Invitrogen), and a 119-bp *TDGF1* fragment was amplified. Two human *TDGF1* oligonucleotide primers for the PCR reaction were designed as follows: 5'-AGATGGCCCGCTTCTCTTAC-3' (forward), 5'-CAGGTATCCCCGAGATGGAC-3' (reverse). The forward primer is located in exon 1 and the reverse primer is located in exon 2. PCR was performed with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGAC TCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. cDNA from the human reference total RNA (Clontech, Palo Alto, CA, USA) was used as a source of positive controls. Real-time PCR monitoring was performed using the Light Cycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for *TDGF1* and *GAPDH* cDNA amplification. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and elongation at 72°C for 10 sec. The products were then subjected to a temperature gradient from 55 to 95°C at 0.1°C $s^{-1}$ with continuous fluores-cence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

*Proliferation and invasion assays.* To assess the cell prolife-ration after 48 h of siRNA transfection, they were grown for another 48 h. The cell viability was determined utilizing Cell Counting kit consisted of WST-8 (Dojin, Tokyo, Japan). WST-8 (10 $\mu$l) was added to the 100 $\mu$l medium containing each supplement above, and the absorbance was read at 450 nm using Microplate Reader (Model 680XR, Bio-Rad Labora-tories, CA). All the experiments were performed at 50-80% cell confluence, and the results were confirmed in five independent experiments. The values were expressed as a ratio/control (every parental cell).

Cell invasion were assessed with CytoSelect Cell Invasion Assay according to the protocol of the manufacture (Cell Biolabs, San Diego, CA) after 48 h of the transfection. Cells ($1.0 \times 10^5$) in DMEM were placed on each 8.0-$\mu$m pore size membrane insert in 96-well plates. DMEM with 10% FBS was placed in the bottom wells. After 24 h, cells that did not invade were removed from the top side of the membrane chamber and completely dislodge the cells from the underside of the membrane by tilting the membrane chamber in the Cell Detachment Solution (Cell Biolabs). Lysis Buffer/CyQuant GR dye solution (Cell Biolabs) were added to each well, the fluorescence of the mixture was read with a fluorescence plate reader at 480/520 nm. The values were expressed as a ratio/control (every parental cell).

*Statistical analysis.* The variable data are expressed as mean ± SEM. The relationship between *TDGF1* expression and clinico-pathological factors was analyzed with the $\chi^2$ test. Kaplan-Meier survival curves were plotted and compared with the generalized log-rank test. Univariate and multivariate analyses were performed to identify prognostic factors using a Cox proportional hazard regression model. The Wilcoxon rank test was used to compare differences in *TDGF1* siRNA among the cell lines. All tests were analyzed with JMP software (SAS Institute, Cary, NC, USA). Differences with p<0.05 were considered statistically significant.

## Results

*Expression of TDGF1 in clinical tissue specimens and clinico-pathological characteristics.* We performed quantitative real-time RT-PCR with paired primary and adjacent non-cancerous CRC regions. RT-PCR on 55 paired clinical samples showed that 17 of these cases (30.9%) exhibited higher levels of *TDGF1* mRNA in tumors than in paired normal tissues. *TDGF1* expression was calculated by dividing *TDGF1*/*GAPDH* expression. For clinicopathological evaluation the experimental samples were divided into 2 groups according to expression status. Patients with values more than the median *TDGF1* expression value (median, 1,960) were assigned to the high-expression group and the others were assigned to the low-expression group. Clinicopathological factors related to the *TDGF1* expression status of the 55 patients are summarized in Table I. The results indicated that *TDGF1* expression was correlated with lymphatic invasion (p=0.041), venous invasion (p=0.010), and metastasis (p=0.052). To examine the corre-lation with metastasis, which indicated a poor prognosis, the data were divided into monochronous and metachronous metastasis groups, and *TDGF1* expression was evaluated for

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Table I. Clinicopathological factors and *TDGF1* mRNA expression in 55 colorectal cancers.

| Factors | High expression n=27 (%) | Low expression n=28 (%) | P-value |
|---|---|---|---|
| Age (years) | | | |
| ≥68 | 11 (40.7) | 16 (57.1) | 0.222 |
| <68 | 16 (59.3) | 12 (42.9) | |
| Gender | | | |
| Male | 14 (51.8) | 17 (60.7) | 0.507 |
| Female | 13 (48.2) | 11 (39.3) | |
| Histological grade | | | |
| Wel/Mod | 23 (85.2) | 25 (89.3) | 0.648 |
| Others | 4 (14.8) | 3 (10.7) | |
| Tumor size | | | |
| ≥50 mm | 10 (37.0) | 17 (60.7) | 0.079 |
| <50 mm | 17 (63.0) | 11 (39.3) | |
| Tumor invasion | | | |
| Tis | 1 (3.7) | 6 (21.4) | 0.051 |
| T1 | 0 (0) | 4 (14.3) | |
| T2 | 6 (22.2) | 5 (17.9) | |
| T3 | 17 (63.0) | 10 (35.7) | |
| T4 | 3 (11.1) | 3 (10.7) | |
| Lymph node metastasis | | | |
| N0 | 17 (66.7) | 20 (71.4) | 0.702 |
| N1-2 | 9 (33.3) | 8 (28.6) | |
| Lymphatic invasion | | | |
| Absent | 4 (14.8) | 11 (39.3) | 0.041 |
| Present | 23 (85.2) | 17 (60.7) | |
| Venous invasion | | | |
| Absent | 11 (40.7) | 21 (75.0) | 0.010 |
| Present | 16 (59.3) | 7 (25.0) | |
| Metastasis | | | |
| M0 | 17 (63.0) | 24 (85.7) | 0.052 |
| M1 | 10 (37.0) | 4 (14.3) | |
| UICC stage | | | |
| 0 | 1 (3.7) | 6 (21.4) | 0.133 |
| I | 4 (14.8) | 8 (28.6) | |
| IIA | 7 (25.9) | 5 (17.8) | |
| IIB | 2 (7.4) | 1 (3.6) | |
| IIIA | 1 (3.7) | 0 (0) | |
| IIIB | 2 (7.4) | 2 (7.1) | |
| IIIC | 0 (0) | 2 (7.1) | |
| IV | 10 (37.0) | 4 (14.3) | |

Statistically significant values are underlined. Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

Table II. Metastasis and *TDGF1* mRNA expression in the 55 patients.

| Factors | High expression n=27 (%) | Low expression n=28 (%) | P-value |
|---|---|---|---|
| Monochronous metastasis | | | |
| Absent | 24 (88.9) | 25 (89.3) | 0.052 |
| Present | 3 (11.1) | 3 (10.7) | |
| Metachronous metastasis | | | |
| Absent | 17 (70.8) | 24 (96.0) | 0.017 |
| Present | 7 (29.2) | 1 (4.0) | |

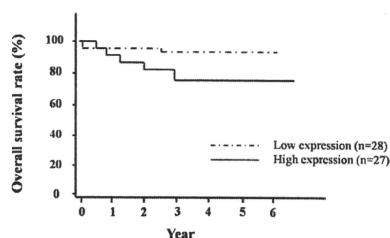Underlined values indicate statistical significance.



Figure 1. Overall survival rates of colorectal cancer patients based on *TDGF1* mRNA expression status. The overall survival rate was lower in the *TDGF1* high-expression group than the low-expression group (p=0.144).
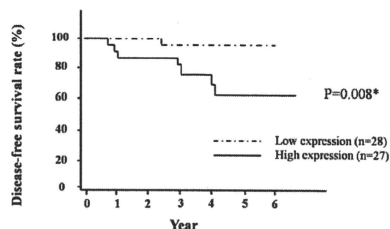


Figure 2. Disease-free survival rates of colorectal cancer patients, exclusive of monochronous metastasis, based on *TDGF1* mRNA expression status. The disease-free survival rate was significantly lower in patients whose samples highly expressed *TDGF1* mRNA than those with lower expression (p=0.008).

each factor (summarized in Table II). The results indicated that *TDGF1* expression was significantly correlated with metachronous metastasis (p=0.017).

*Relationship between TDGF1 expression and prognosis.* Postoperative overall survival was shorter in patients with elevated *TDGF1* expression (p=0.144) than in those with lower expression. The median follow-up was 4.16 years (Fig. 1). We also evaluated disease-free survival based on the relationship between *TDGF1* expression and metachronous metastasis after

Table III. Univariate and multivariate analyses for disease-free survival in 49 patients with curative surgery (Cox proportional hazards regression model).

| Factors | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | RR | 95% CI | P-value | RR | 95% CI | P-value |
| Age (years) | | | | | | |
| ≤68/>68 | 1.84 | 0.45-9.01 | 0.391 | | | |
| Gender | | | | | | |
| Male/female | 2.17 | 0.62-18.62 | 0.192 | | | |
| Histological grade | | | | | | |
| Por-others/well-mod | 713.31 | - | 0.241 | | | |
| Tumor size (mm) | | | | | | |
| ≥50/<50 | 3.34 | 0.76-22.91 | 0.110 | | | |
| Tumor invasion | | | | | | |
| T3-4/Tis-2 | 3.02 | 0.69-20.70 | 0.145 | | | |
| Lymph node metastasis | | | | | | |
| N1-2/N0 | 4.21 | 0.99-17.85 | 0.051 | | | |
| Lymphatic invasion | | | | | | |
| Present/absent | - | - | <u>0.014</u> | - | - | 0.067 |
| Venous invasion | | | | | | |
| Present/absent | 2.53 | 0.59-10.72 | 0.196 | | | |
| *TDGF1* mRNA expression | | | | | | |
| < Median/≥ median | 10.42 | 1.84-195.08 | <u>0.005</u> | 7.78 | 1.37-146.02 | <u>0.017</u> |

Statistically significant values are underlined. RR, relative risk; CI, confidence interval; Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.
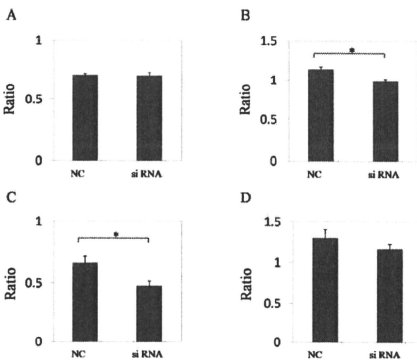


Figure 3. *In vitro* assays with siRNA inhibition in the two colorectal cancer cell lines. A proliferation assay was performed in two colorectal cancer cell lines (A, HCT116; B, LoVo). There were significant differences between NC and *TDGF1* siRNA in LoVo (n=5, *p=0.008). An invasion assay was performed in them (C, HCT116; D, LoVo). There were significant differences between NC and *TDGF1* siRNA in HCT116 (n=5, *p=0.009). *In vitro* assays showed differences in the ratio with control (untreated) cells. Values are means and SEM. NC, negative control.

curative surgery in 49 patients except stage IV at the time of primary operation. The disease-free survival rate was significantly lower in patients with elevated *TDGF1* expression (p=0.008; Fig. 2) than in those with lower expression. Table III shows the univariate and multivariate analyses of factors related to metastatic-free survival in 49 patients. The univariate analysis revealed that *TDGF1* expression (p=0.005) and lymphatic invasion (more than maximum repetition, p=0.014) were significantly correlated with post-operative metastasis. The multivariate regression analysis indicated that inclusion in the *TDGF1* high-expression group (relative risk, 7.78; 95% confidence interval, 1.37-146.02; p=0.017) was an independent predictor of metastatic-free survival.

*In vitro assessment of TDGF1 expression knock-down.* Two CRC cell lines were chosen for the proliferation and invasion study. A significant reduction in *TDGF1* by siRNA was also confirmed by quantitative real-time RT-PCR. The proliferation study was confirmed by seeding the cells ($1.0 \times 10^5$) in 6-well dishes and culturing them for 48 h to determine proliferation. The results showed significant differences in HCT116 and LoVo cell numbers between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3A and B). In the invasion study, the results showed significant differences in DLD-1 and LoVo between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3C and D).

## Discussion

*TDGF1*, also known as *CRYPTO*, Crypto-1, or CR-1, is expressed in various cancer tissues of different species (8-16,20-23). Previous *in vitro* and *in vivo* reports show that *TDGF1* regulates signaling pathways and cellular mechanisms as an oncogene (23-26). In mammary tumor, *TDGF1* is associated with molecular mechanisms that contribute to the loss of adherent junctions, referred to as epithelial-mesenchymal transition, which plays an important role in cancer invasiveness and metastasis and might cause a poor prognosis (25-28). The combined expression of *TDGF1* and E-cadherin by immunohistochemistry indicates a poor prognosis in gastric cancer (16).

The present study showed that *TDGF1* expression is an independent predictive factor for metachronous CRC metastasis, and the siRNA inhibition experiment demonstrated functional relevance of expressed *TDGF1* in the CRC cell lines. To the best of our knowledge, this is the first report to show that *TDGF1* is a predictive marker for CRC metastasis, supported by the functional relevance to cell growth and invasion.

It can be useful to identify the necessity for intensive follow-up and adjuvant therapy by predicting CRC recurrence and metastases after curative surgical resection (29-31). Our clinicopathological analysis revealed that CRC patients with high *TDGF1* expression had a poorer prognosis for disease-free survival than the low-expression group. The results indicated that *TDGF1* is a good predictor for metachronous metastasis, and patients can be followed-up by curative surgical intervention. It is essential to prevent metachronous metastasis during gastrointestinal cancer therapy. Several adjuvant chemotherapies are helpful in particular disease stages, especially in CRC, and indicate the usefulness of a less invasive surgical approach for CRC (31-36). For these cases, a predictive informative marker for tumor recurrence, which is independent from traditional TNM classification and collectively contributes to diagnoses and treatments is very important. While improvement in preoperative and postoperative treatments such as chemotherapy and radiotherapy combined with surgery have contributed to a reduction in the recurrence and metastasis of CRC, half of the cases ultimately metastasize despite systemic chemotherapy followed by surgery (37). Adjuvant chemotherapy for CRC is desirable in highly suspicious metastatic cases. In these cases, an analysis of *TDGF1* may be useful to predict and treat patients with a poor prognosis.

## Acknowledgements

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# Phase II study of erlotinib plus gemcitabine in Japanese patients with unresectable pancreatic cancer

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Erlotinib combined with gemcitabine has not been evaluated in Japanese patients with unresectable pancreatic cancer. This two-step phase II study assessed the safety and pharmacokinetics of erlotinib 100 mg/day (oral) plus gemcitabine 1000 mg/m² (i.v. days 1, 8, 15) in a 28-day cycle in the first step, and efficacy and safety in the second step. The primary end-point was safety. One hundred and seven patients were enrolled (first step, $n = 6$; second step, $n = 101$). The most common adverse event was RASH (compiled using the preferred terms rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash) in 93.4% of patients. One treatment-related death occurred. While interstitial lung disease-like events were reported in nine patients (8.5%; grade 1/2/3, 3.8/2.8/1.9%), all patients recovered or improved. The median overall survival, the 1-year survival rate and median progression-free survival were 9.23 months, 33.0% and 3.48 months, respectively. The overall response and disease control rates were 20.3% and 50.0%, respectively. In Japanese patients with unresectable pancreatic cancer, erlotinib plus gemcitabine had acceptable toxicity and efficacy that was not inferior to that seen in Western patients. (Cancer Sci 2011; 102: 425–431)

A pproximately 232 000 individuals are diagnosed with pancreatic cancer worldwide each year, with an annual death rate estimated at 227 000.[1] In Japan, approximately 22 000 new cases were reported in 2005.[2] Furthermore, data from 2007 show that around 24 000 individuals in Japan died from pancreatic cancer, making this tumor type the fifth leading cause of cancer-related death.[3] The majority of pancreatic cancer cases are diagnosed at an unresectable stage when prognosis is extremely poor.

Current treatment for advanced pancreatic cancer is based on systemic chemotherapy with gemcitabine. Single-agent gemcitabine has been shown to extend median overall survival (OS) to 5.65 months in chemonaïve patients compared with 4.41 months in patients who received fluorouracil.[4] Addition of other cytotoxic agents to gemcitabine has not demonstrated survival benefits over gemcitabine alone.[5–13] The potential of combining gemcitabine with biological agents in patients with advanced pancreatic cancer has also been evaluated in several phase III studies, but these trials failed to show a survival benefit.[14–19]

Epidermal growth factor receptor (EGFR)-mediated signaling is associated with various cellular processes, and the dysregulation of these processes is common in tumorigenesis.[20,21] Furthermore, EGFR is overexpressed in many tumors and its

overexpression is often associated with poor prognosis.[22–26] EGFR tyrosine-kinase inhibitors (TKI, such as erlotinib) are used in the treatment of various types of solid tumors.

Erlotinib has demonstrated antitumor activity in pancreatic cell lines[27] and was subsequently assessed as a potential therapeutic agent in pancreatic cancer. In the PA.3 study ($n = 569$), the risk of death with erlotinib plus gemcitabine was reduced by 18% versus gemcitabine alone (hazard ratio [HR], 0.82; 95% confidence interval [CI], 0.69–0.99; $P = 0.038$ after adjustment for stratification factors), with a median OS of 6.24 months *vs* 5.91 months, respectively. Erlotinib plus gemcitabine combination therapy provided significant improvements in the 1-year survival rate (23% *vs* 17%; $P = 0.023$) and progression-free survival (PFS; HR 0.77; 95% CI, 0.64–0.92; $P = 0.004$).[28] As a result, this combination was approved for use in pancreatic cancer in many countries.

In Japanese patients with non-small-cell lung cancer (NSCLC), a phase II study has specifically shown that erlotinib monotherapy is well tolerated and has promising antitumor activity.[29] However, there are no data on the use of erlotinib combined with gemcitabine in Japanese patients with pancreatic cancer. This phase II study evaluated the safety and efficacy of erlotinib in combination with gemcitabine in Japanese patients with unresectable locally advanced or metastatic pancreatic cancer.

## Methods

**Patients.** Patients aged 20–80 years with histological/cytological evidence of unresectable locally advanced or metastatic adenocarcinoma/adenosquamous carcinoma of the pancreas were eligible for inclusion in the present study. Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2, adequate hematological, renal and hepatic function and a life expectancy of at least 2 months. No more than one prior regimen for pancreatic cancer was permitted. Patients who had received prior gemcitabine and/or a TKI were excluded from participation, as were those who had previously been exposed to a human epidermal growth factor receptor 2 (HER2) or EGFR inhibitor. Other key

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exclusion criteria were: symptomatic cerebral metastases; a concurrent lung disorder (such as idiopathic pulmonary fibrosis, interstitial lung disease [ILD] or pneumoconiosis); concurrent or previous drug-induced pneumonia; or a history of radiation to the chest.

The study complied with the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all patients, and the protocol was approved by ethics committees at all participating institutions.

**Study design and treatment.** This was a phase II, multicentre, open-label, two-step study. In the first step, six patients were enrolled into the study and treated with oral erlotinib 100 mg/day on days 3–28, plus i.v. gemcitabine 1000 mg/m$^2$ on days 1, 8 and 15 in a 28-day cycle. The starting doses of erlotinib and gemcitabine were chosen in reference to the PA.3 study. Dose-limiting toxicities (DLT) were assessed in these study participants using the National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 (NCI-CTCAE, National Cancer Institute, Bethesda, MD, USA). Dose-limiting toxicities were defined in conformity to the P1b study as follows:[30] (i) grade 4 decrease (i.e. to <500/mm$^3$) in neutrophil count >5 days; (ii) grade ≥3 decrease (i.e. to <1000/mm$^3$) in neutrophil count with associated fever (≥38.5°C); (iii) grade 4 decrease in platelet count (i.e. to <25 000/mm$^3$); (iv) any grade ILD; (v) grade 4 elevation of alanine transaminase (ALT)/aspartate transaminase (AST) levels, or grade 3 elevation of ALT/AST levels >7 days; (vi) grade ≥3 non-hematological toxicity (excluding rash, hyperglycemia, γ-GTP and events that were judged to be transient/had no effect on study continuation); and (vii) grade 4 dose-reduction/interruption required due to persistent adverse events (AE), which meant that the second cycle could not be started.

If treatment-related DLT occurred in no more than two of the six patients, transition to the second step of the study was permissible with approval of the Data Safety and Monitoring Committee (DSMC). If DLT occurred in three or more patients, transition to the second step was limited to those cases that were judged to be safe for this study after the DSMC had evaluated the safety data of the patients with a DLT. In the second step, it was planned that 94 patients would be treated with the same dose as the first step. Treatment was continued until disease progression, death, unacceptable toxicity or patient/investigator request.

The primary end-point of the study was safety, with secondary end-points including OS, 1-year survival rate, PFS, overall response rate (ORR), disease control rate (DCR = complete response [CR] + partial response [PR] + stable disease), pharmacokinetics (PK) and correlation of *EGFR* mutation status with outcomes.

**Toxicity evaluation.** Adverse events were monitored and graded using NCI-CTCAE v3.0. Clinical and laboratory assessments were conducted throughout the study. Adverse events prespecified in the study to be monitored carefully were rash, diarrhea, vomiting, liver dysfunction and ILD-like events. Chest X-ray examination to assess pulmonary toxicity was conducted weekly until week 4 and every 2 weeks thereafter. In addition, chest computed tomography (CT) scan was performed every 4 weeks. The DSMC reviewed the images and clinical data associated with all potential ILD-like events. All ILD-like events were reported to be serious AE (SAE), regardless of grade.

**Efficacy evaluation.** The tumor response was assessed using Response Evaluation Criteria in Solid Tumors (RECIST) in patients who had at least one measurable target lesion. Tumors were measured using computed tomography (CT) at baseline and on day 22 of every two cycles thereafter. Median PFS, ORR and DCR were estimated by the extramural review. The relationship between efficacy and the severity of RASH (compiled

using the preferred terms rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash) was also examined.

**Pharmacokinetic evaluation.** Pharmacokinetic evaluation of erlotinib and its O-desmethylated metabolite (OSI-420) was performed in the six patients enrolled in the first step of the study. Venous blood samples were taken prior to erlotinib dosing on day 3 and day 8 of cycle 1 at 0.5, 1, 2, 4, 6, 8 and 24 h after erlotinib administration. Samples were also taken prior to gemcitabine infusion on days 1 and 8 at 0.5, 0.75, 1, 1.5, 2.5 and 4.5 h after dosing.

The plasma concentrations of erlotinib, OSI-420 and gemcitabine were measured by liquid chromatography, tandem mass spectrometry (LC-MS-MS). The LC-MS-MS analytical methods have been described previously.[31,32] Derived PK parameters included the maximum plasma drug concentration ($C_{max}$), time to $C_{max}$ ($t_{max}$), area under the plasma drug concentration-time curve to the last plasma sample (AUC$_{last}$), terminal half-life ($t_{1/2}$) and oral clearance (CL/F).

**Biomarker analysis.** *EGFR* mutations were assessed in patients with available tumor tissue specimens, which were formalin fixed and paraffin embedded. Samples were analyzed at a central laboratory where DNA was extracted and exons 18–21 sequenced using a nested PCR.

**Statistical analysis.** Progression-free survival and OS were estimated using the Kaplan–Meier method in all patients who received at least one dose of the study treatment, with 95% CI for the median duration calculated using Greenwood's formula. The Clopper–Pearson method was used to calculate the 95% CI around the ORR, DCR and AE rate. Multivariate analyses were performed for the occurrence of ILD-like events using the logistic regression model. Baseline characteristics investigated for this analysis included gender, age, lung metastasis, emphysema and various baseline laboratory values. The target enrollment was 100 patients, as this was required to evaluate the safety of erlotinib.

## Results

**Patient characteristics.** Between December 2006 and October 2007, a total of 107 patients were enrolled (first step, $n = 6$; second step, $n = 101$) from 12 institutions (Fig. 1). One patient who enrolled into the second step did not receive treatment due to deterioration in PS prior to the start of treatment. A total of 106 patients were evaluable for safety (safety population, full analysis set).

The patient demographics and baseline characteristics are shown in Table 1. The median age was 62 years (range, 36–78) and 52.8% of patients were male. Almost all patients were chemonaïve (95.3%). The majority (75.5%) of patients had an ECOG PS of 0 and most (83.0%) had metastatic disease. Over half (63.2%) of the patients had a history of current or past smoking.

**Toxicity and dose modifications.** The median duration of erlotinib exposure was 102.5 days and its median dose intensity was 100.0 mg/day, with the majority of patients (78.3%) receiving more than 90% of the relative dose intensity. The median duration of gemcitabine treatment was 4.0 cycles and its median dose intensity was 688.0 mg/m$^2$ per week, with approximately half of the patients (51.4%) receiving more than 90% of the relative dose intensity.

As only one patient had a DLT (grade 3 diarrhea) in the first step, the second step of the study was initiated. One hundred and six patients received at least one dose of erlotinib; these patients were assessable for toxicity. Treatment-related AE and treatment-related changes in laboratory values are summarized in Table 2; most of these were mild to moderate in severity. The most frequently reported AE was RASH, which occurred in
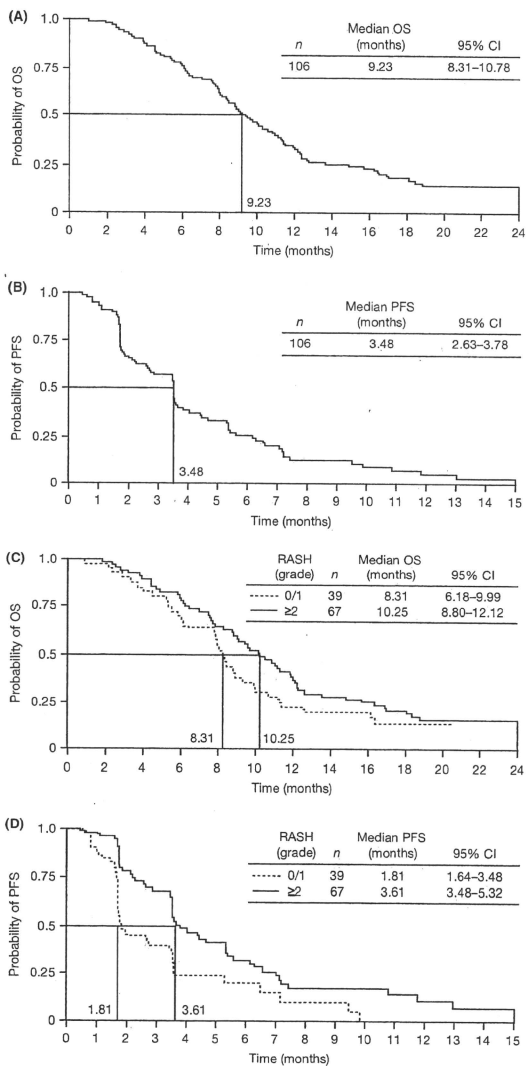
**Fig. 1.** Kaplan–Meier estimates of (A) overall survival (OS) and (B) progression-free survival (PFS) in the study population (n = 106); (C) OS and (D) PFS according to the severity of RASH (grade ≤1 [n = 39] vs grade ≥2 [n = 67]). RASH is a composite of the terms: rash, acne, exfoliative rash, dermatitis acneiform, erythema, eczema, dermatitis and pustular rash. CI, confidence interval.

**Table 1. Baseline characteristics and demographics (n = 106)**

| Characteristic | |
| --- | --- |
| Median age (range) (years) | 62 (36–78) |
| Gender, n (%) | |
| Male | 56 (52.8) |
| Female | 50 (47.2) |
| Median bodyweight (range) (kg) | 52.3 (33.1–95.0) |
| Smoking history,† n (%) | |
| Never smoker | 39 (36.8) |
| Past smoker | 37 (34.9) |
| Current smoker | 30 (28.3) |
| ECOG PS, n (%) | |
| 0 | 80 (75.5) |
| 1 | 26 (24.5) |
| 2 | 0 (0) |
| Disease status, n (%) | |
| Metastatic | 88 (83.0) |
| Locally advanced | 18 (17.0) |
| Primary tumor identified, n (%) | 92 (86.8) |
| Primary sites, n (%) | |
| Head | 46 (43.4) |
| Body and tail | 23 (21.7) |
| Body | 22 (20.8) |
| Tail | 10 (9.4) |
| Other | 5 (4.7)‡ |
| Biliary drainage, n (%) | 19 (17.9) |
| Sites of distant metastases, n (%) | |
| Liver | 56 (52.8) |
| Distant lymph nodes | 39 (36.8) |
| Lung | 17 (16.0) |
| Other | 26 (24.5) |
| Prior lines of therapy, n (%) | |
| None | 101 (95.3) |
| One regimen | 5 (4.7)§ |
| Median CA19–9 (range) (U/mL) | |
| Median | 776 (0–435 000) |
| Median CEA (range) (ng/mL) | |
| Median | 4.8 (0.6–1100.1) |

†Never smoker, never/hardly smoked; past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). ‡Whole of pancreas (n = 1); head and body (n = 3); other (n = 1). §Tegafur, gimeracil, oteracil potassium (S-1) (n = 3); 5-fluorouracil plus leucovorin (n = 2). CA 19–9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; ECOG, Eastern Co-Operative Group.

**Table 2. Treatment-related adverse events occurring in >30% of patients treated with erlotinib and gemcitabine (n = 106)**

| | Any grade, n (%) | Grade 3, n (%) | Grade 4, n (%) |
| --- | --- | --- | --- |
| Non-hematological | | | |
| Rash | 78 (73.6) | 3 (2.8) | 0 (0) |
| Anorexia | 75 (70.8) | 15 (14.2) | 0 (0) |
| Pruritus | 57 (53.8) | 1 (0.9) | 0 (0) |
| Fatigue | 56 (52.8) | 3 (2.8) | 0 (0) |
| Nausea | 56 (52.8) | 6 (5.7) | 0 (0) |
| Diarrhea | 52 (49.1) | 2 (1.9) | 0 (0) |
| Dry skin | 49 (46.2) | 0 (0) | 0 (0) |
| Stomatitis | 38 (35.8) | 0 (0) | 0 (0) |
| Pyrexia | 32 (30.2) | 0 (0) | 0 (0) |
| Hematological | | | |
| White blood cell count decreased | 85 (80.2) | 31 (29.2) | 0 (0) |
| Platelet count decreased | 77 (72.6) | 9 (8.5) | 0 (0) |
| Hemoglobin decreased | 76 (71.7) | 13 (12.3) | 2 (1.9) |
| Hematocrit decreased | 73 (68.9) | 8 (7.5) | 0 (0) |
| Neutrophil decreased | 73 (68.9) | 32 (30.2) | 5 (4.7) |
| Red blood cell count decreased | 72 (67.9) | 8 (7.5) | 0 (0) |
| ALT increased | 59 (55.7) | 10 (9.4) | 0 (0) |
| AST increased | 57 (53.8) | 4 (3.8) | 1 (0.9) |
| Weight decreased | 53 (50.0) | 3 (2.8) | 0 (0) |
| Lymphocyte count decreased | 46 (43.4) | 14 (13.2) | 0 (0) |
| Blood albumin decreased | 35 (33.0) | 0 (0) | 0 (0) |
| Gamma-glutamyltransferase increased | 35 (33.0) | 12 (11.3) | 1 (0.9) |

ALT, alanine amino transferase; AST, aspartate amino transferase.

93.4% of the patients; most cases were mild to moderate in severity (87.7%, grade ≤2; 5.7%, grade ≥3). Other common non-hematological AE included anorexia, pruritus, fatigue, nausea and diarrhea. Most patients experienced some degree of hematological toxicity, with grade 3 or 4 neutropenia (neutrophil decreased), leucopenia (white blood cell count decreased) and anemia (hemoglobin decreased) occurring in 34.9%, 29.2% and 14.2% of patients, respectively. Only one treatment-related death occurred (due to gastrointestinal hemorrhage), which was probably due to arterial bleeding caused by the invasion of the primary tumor into the gastrointestinal tract. Although the likelihood of this event being treatment-related was deemed remote, a causal relationship could not be completely excluded because the event occurred during the study treatment administration period.

Treatment-related SAE were reported in 26 (24.5%) patients. These included nine ILD-like events (8.5%), the majority of which (n = 7) were grade 1–2 in severity. Importantly, all of these nine patients recovered or improved, and four of these patients did so without any treatment for ILD-like events. Other

treatment-related SAE were anorexia (3.8%), vomiting, pyrexia and abnormal hepatic function (1.9% each). The baseline characteristics, treatment and outcomes of patients who developed treatment-related ILD-like events during the study are detailed in Table 3. The onset times of ILD-like events ranged from 7 to 187 days after the start of treatment. In these patients, a relatively long survival was observed (from 119 to 568+ days), and five patients received post-study therapy. All of these nine patients were past or current smokers, and six had emphysema at baseline (not detected prior to treatment, but diagnosed at the extramural review by a radiologist in the DSMC). Multivariate analyses were performed for the occurrence of ILD-like events using the logistic regression model and emphysema at baseline was indicated as a risk factor for onset of ILD-like events (odds ratio [95% CI], 12.13 [1.01–145.7]; $P = 0.0491$).

Adverse events led to erlotinib discontinuation in 30 patients (28.3%) and gemcitabine discontinuation in 27 patients (25.5%). The main reasons for treatment discontinuation were ILD (n = 6) and anorexia (n = 3); no patient discontinued treatment due to RASH or diarrhea. Due to the onset of AE, a total of 65 patients (61.3%) required one or more interruptions of erlotinib (36 patients [34.0%] for longer than seven consecutive days and 17 patients [16.0%] for longer than 14 consecutive days) and 56 patients (52.8%) had one or more skip of gemcitabine. Modifications in the erlotinib or gemcitabine dosage were required in 17 (16.0%) and 11 (10.4%) patients, respectively, due to AE.

**Efficacy.** The median OS was 9.23 months (95% CI, 8.31–10.78; Fig. 1A) and the 1-year survival rate was 33% (95% CI, 24–42). Median PFS was 3.48 months (95% CI, 2.63–3.78; Fig. 1B). Among these 106 patients of ILD-like events tumor response (n = 64), the ORR was 20.3% (13/64; 95% CI, 11.3–32.2) and the DCR was 50.0% (95% CI, 37.2–62.8; CR, n = 0; PR, n = 13; stable disease, n = 19).

Table 3. Characteristics, treatment and outcomes of patients with treatment-related ILD-like events ($n = 9$)

| Event | Gender | Age (years) | Smoking status† | Days on treatment | ILD maximum grade | Suspicious findings of ILD | Steroids | Oxygen | ILD outcome | Presence of emphysema (assessed by radiologist) | Survival outcome (days) | Post-therapy (chemotherapy) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lymphoid ILD | M | 62 | Past | 82 | 1 | Pyrexia | None | No | Improved | Yes | 362 | Yes |
| ILD | M | 42 | Current | 50 | 3 | Pyrexia | Pulse | Yes | Recovered | Yes | 517 | Yes |
| Organising pneumonia | M | 60 | Past | 183 | 2 | Respiratory symptoms | None | No | Improved | Yes | 568+ | Yes |
| ILD | F | 62 | Past | 113 | 2 | Cough | Oral | No | Recovered | Yes | 376 | No |
| ILD | F | 74 | Past | 111 | 3 | Cough, dyspnea | Pulse | Yes | Improved | None | 183 | No |
| ILD | M | 60 | Current | 25 | 1 | Pyrexia | Pulse | No | Recovered | None | 119 | Yes |
| ILD | M | 77 | Past | 7 | 1 | X-ray | None | No | Recovered | Yes | 255 | No |
| ILD | M | 55 | Past | 187 | 1 | CT | None | No | Recovered | Yes | 415 | No |
| ILD | F | 60 | Current | 76 | 2 | Cough | Oral | No | Recovered | None | 346 | Yes |

†Past smoker, passage of at least 1 month since stopping smoking (at the time of registration); current smoker, smoked within 1 month (at the time of registration). CT, computed tomography; F, female; ILD, interstitial lung disease; M, male.

The median OS was longer in patients who experienced RASH of grade ≥2 ($n = 67$) than in those with RASH of grade ≤1 ($n = 39$) (10.25 months [95% CI, 8.80–12.12] vs 8.31 months [95% CI, 6.18–9.99], respectively; Fig. 1C) and the 1-year survival rate was higher (39% [95% CI, 27–50] vs 23% [95% CI, 10–36], respectively). Similarly, the median PFS was longer in patients with RASH of grade ≥2 versus those with RASH grade ≤1 (3.61 months [95% CI, 3.48–5.32] vs 1.81 months [95% CI, 1.64–3.48]; Fig. 1D). While there was no notable difference in ORR between patients with RASH grade ≥2 and those with grade ≤1 (21.1% [95% CI, 9.6–37.3] vs 19.2% [95% CI, 6.6–39.4]), the DCR was higher in those with more severe RASH (60.5% [95% CI, 43.4–76.0] vs 34.6% [95% CI, 17.2–55.7]).

**Pharmacokinetics.** Plasma sampling for PK analyses was performed in all six patients enrolled in the first step. On day 8, the values of $C_{max}$ were 1760 ± 456.9 ng/mL (mean ± SD) for erlotinib, 169.7 ± 64.5 ng/mL for OSI-420 and 22 700 ± 3272.9 ng/mL for gemcitabine. The AUC$_{last}$ was 29 001 ± 6560 h ng/mL, 2748 ± 788 h ng/mL and 10 717 ± 1458 h ng/mL (mean ± SD), respectively. The mean $t_{max}$ was 8.0 h (range, 2.0–23.9 h), 9.0 h (2.0–23.9 h) and 0.51 h (0.45–0.57 h), respectively. Also on day 8, the mean plasma $t_{1/2}$ was 54.92 h (range, 9.25–144.61 h), 32.79 h (10.36–60.46 h), and 0.63 h (0.31–1.14 h), respectively. The Cl/F of erlotinib and gemcitabine showed interindividual variability; the Cl/F on day 8 was 3972.9 ± 772.1 mL/h (mean ± SD; coefficient of variation 19.4%) and 146 580.4 ± 31 101.3 mL/h (21.2%), respectively.

**Biomarker analysis.** Of the 106 patients enrolled, *EGFR* mutation status was evaluated in 47 patients (44.3%), all of whom had wild-type *EGFR*. The mutation status of the remaining patients was classified as unknown because samples not available (30.2%), not examined (9.4%) or the results following sequencing were inconclusive (16.0%).

## Discussion

This study was designed to initially assess the safety of erlotinib with gemcitabine for Japanese patients with pancreatic cancer, in whom there had been no prior exposure to either drug. As no significant safety concerns were raised in the first step of the study, enrollment of a further 101 patients was performed. Although the incidence of AE in this study was higher than in the PA.3 study, the incidence of grade 3–4 AE was similar.[28] Despite these results, no new AE specific to Japanese patients

were observed. As expected, RASH and gastrointestinal events were among the most common AE in this study, and most of these cases were mild to moderate in severity.

Interstitial lung disease-like events were reported in nine patients (8.5%; grade 1/2/3, 3.8/2.8/1.9%) in the current study, while its incidence was reported to be 2.4% in patients treated in the erlotinib plus gemcitabine arm of the PA.3 study.[28] In addition, in Japanese patients with advanced pancreatic cancer, ILD-like events were reported in two (6.1%) of 33 patients treated with gemcitabine plus S-1, and were reported in three (1.1%) of 264 patients with gemcitabine monotherapy, respectively.[33,34] Likewise, the higher incidence of ILD-like events were documented using S-1 or erlotinib in combination with gemcitabine compared with gemcitabine as monotherapy in patients with pancreatic and biliary tract cancer.[35] On another front, outside of Japan, a high incidence of ILD-like events was reported in gemcitabine and paclitaxel combination therapy in patients with NSCLC.[36] From the above information, considering the higher incidence of ILD when gemcitabine is used in combination, an additive effect from such combinations cannot be ruled out.

In NSCLC, Japanese patients have an increased risk of developing ILD-like events when treated with EGFR TKI.[29,37–39] Fatal cases of ILD-like events have been reported following EGFR TKI administration for the treatment of NSCLC.[37–41] Importantly, however, no patients died due to an ILD-like event in this study. Seven patients experienced ILD-like events of grade 1–2 in severity. This may be due to active management of ILD-like cases during the study period. This management included regular and immediate chest X-rays, in addition to diagnosis with CT scans after any early signs and symptoms were observed (e.g. pyrexia, cough or dyspnea), timely discontinuation of the antitumor drugs (as a precautionary measure in case these drugs were associated with the symptoms) and appropriate treatment for the events (including oral/pulse steroids). By appropriately treating the early symptoms of ILD-like events, patients could restart antitumor therapy (chemotherapy: treatment change). In this study, the onset time for ILD-like events varied markedly between patients (7–187 days). It is therefore necessary to monitor the patients throughout the treatment period.

All of the patients who developed ILD in this study were current or past smokers, and smoking status has been shown to be a risk factor for ILD in the NSCLC population.[38] Results from the multivariate analyses in this study suggest that emphysema is also a risk factor for developing ILD; six of the nine

patients with ILD-like events were diagnosed with emphysema at baseline. Although the number of reports of an ILD-like event may have been artificially elevated due to underlying patient baseline characteristics and the active management of ILD-like events, these results demonstrate the need to consider the risk of ILD-like events in Japanese patients treated with TKI. In particular, it is important that chest CT scans are closely checked for the presence of emphysema or comorbid ILD and that pulmonary status is assessed prior to treatment administration.

This study corroborates the results of the combination of gemcitabine and erlotinib shown in the PA.3 study. The median OS in this study of 9.23 months was longer than those reported in trials with gemcitabine alone. In this study, patients who experienced skin toxicity of grade ≥2 had better outcomes than those with less severe toxicity or the overall study population. Retrospective analyses of data from the PA.3 and AViTA studies have found a significant association between the development of skin toxicity and efficacy in patients with pancreatic cancer treated with erlotinib-based therapy, although the precise mechanisms for the association between skin toxicity and effectiveness are unknown.[28,41,42]

Although the presence of mutations in the tyrosine-kinase region of the *EGFR* gene appears to predict a better response to erlotinib in NSCLC,[43,44] this has not yet been evaluated in pancreatic cancer. *EGFR* mutations are very rare in patients with pancreatic cancer;[45–47] indeed in the present study, no *EGFR* mutations were detected. Further work is required to determine whether *EGFR* mutations can be used as predictive markers for

improved survival in Japanese patients receiving erlotinib and gemcitabine as treatment for advanced pancreatic cancer.

In conclusion, the present study shows that erlotinib in combination with gemcitabine is generally well tolerated in Japanese patients with advanced pancreatic cancer. This combination is associated with efficacy and survival outcomes, and the results of this study are consistent with the findings of the global PA.3 study.

## Acknowledgments

## Disclosure Statement

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# Adjuvant Chemotherapy With Fluorouracil Plus Folinic Acid vs Gemcitabine Following Pancreatic Cancer Resection
## A Randomized Controlled Trial

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**Context** Adjuvant fluorouracil has been shown to be of benefit for patients with resected pancreatic cancer. Gemcitabine is known to be the most effective agent in advanced disease as well as an effective agent in patients with resected pancreatic cancer.

**Objective** To determine whether fluorouracil or gemcitabine is superior in terms of overall survival as adjuvant treatment following resection of pancreatic cancer.

**Design, Setting, and Patients** The European Study Group for Pancreatic Cancer (ESPAC)-3 trial, an open-label, phase 3, randomized controlled trial conducted in 159 pancreatic cancer centers in Europe, Australasia, Japan, and Canada. Included in ESPAC-3 version 2 were 1088 patients with pancreatic ductal adenocarcinoma who had undergone cancer resection; patients were randomized between July 2000 and January 2007 and underwent at least 2 years of follow-up.

**Interventions** Patients received either fluorouracil plus folinic acid (folinic acid, 20 mg/m², intravenous bolus injection, followed by fluorouracil, 425 mg/m² intravenous bolus injection given 1-5 days every 28 days) (n=551) or gemcitabine (1000 mg/m² intravenous infusion once a week for 3 of every 4 weeks) (n=537) for 6 months.

**Main Outcome Measures** Primary outcome measure was overall survival; secondary measures were toxicity, progression-free survival, and quality of life.

**Results** Final analysis was carried out on an intention-to-treat basis after a median of 34.2 (interquartile range, 27.1-43.4) months' follow-up after 753 deaths (69%). Median survival was 23.0 (95% confidence interval [CI], 21.1-25.0) months for patients treated with fluorouracil plus folinic acid and 23.6 (95% CI, 21.4-26.4) months for those treated with gemcitabine ($\chi^2_1$=0.7; $P$=.39; hazard ratio, 0.94 [95% CI, 0.81-1.08]). Seventy-seven patients (14%) receiving fluorouracil plus folinic acid had 97 treatment-related serious adverse events, compared with 40 patients (7.5%) receiving gemcitabine, who had 52 events ($P$<.001). There were no significant differences in either progression-free survival or global quality-of-life scores between the treatment groups.

**Conclusion** Compared with the use of fluorouracil plus folinic acid, gemcitabine did not result in improved overall survival in patients with completely resected pancreatic cancer.

**Trial Registration** clinicaltrials.gov Identifier: NCT00058201

P ANCREATIC CANCER IS ONE OF the major causes of cancer death globally, with a 5-year survival rate of less than 5%.[1,2] The outlook for those patients who can undergo surgical resection is better, and

in specialized centers, resection rates greater than 15% can be achieved.[3] Although surgery cannot guarantee a cure, the 5-year survival does improve to around 10% following resection.[3] There is a clear need to improve long-term

**See also p 1124 and Patient Page.**

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survival in these patients. While the added survival benefit of adjuvant chemoradiotherapy with or without maintenance chemotherapy[4-7] remains unclear,[8] a more certain survival benefit has been demonstrated from adjuvant chemotherapy.[6,9-14]

The European Study Group for Pancreatic Cancer (ESPAC)-3 trial was designed to compare the survival benefit of adjuvant fluorouracil plus folinic acid vs gemcitabine, which during the conduct of the ESPAC-1 trial had become established as the standard care for advanced pancreatic cancer.[15] Initially this was a 3-group study that included an observation group based on the survival uncertainty of adjuvant chemotherapy[6]; however, the observation group was removed from the design following the definitive results of ESPAC-1.[12] In 2007, the Charité Onkologie Clinical Studies in GI Cancer (CONKO)-001 trial reported improved disease-free survival in patients randomized to receive adjuvant gemcitabine compared with those randomized to receive surgery alone.[13] With 1088 patients randomized, the ESPAC-3 trial represents the largest-ever adjuvant trial conducted in pancreatic cancer, to our knowledge, and results are presented herein.

## METHODS
### Patients and Trial Design
The ESPAC-3 trial was initially introduced as a 3-group study designed to compare the survival benefit of resection alone (observation) with either adjuvant fluorouracil plus folinic acid or gemcitabine. The first patient was entered on July 7, 2000. Following the definitive results from ESPAC-1,[12] the recommendation of the independent data and safety monitoring committee to cease randomization into the control group was adopted on June 20, 2003. The trial design of ESPAC-3 (version 2) therefore necessitated removal of the control group from the original ESPAC-3 (version 1) trial design. ESPAC-3 (version 2) is thus a 2-group, international, open-label,

phase 3, randomized controlled study of adjuvant chemotherapies comparing fluorouracil plus folinic acid with gemcitabine.

The trial was approved by ethics committees at the national and local level according to the requirements of each participating country. All patients entered into the study provided written informed consent following a full explanation of the study and reading of the patient information sheet. There were 159 centers in 17 countries: Australia and New Zealand (26), Canada (15), Czech Republic (1), Finland (1), France (15), Germany (13), Greece (3), Hungary (2), Ireland (2), Italy (3), Japan (7), Poland (1), Serbia (1), Sweden (8), Switzerland (1), and the United Kingdom (60).

### Surgery and Eligibility
Patients were eligible if they had undergone complete macroscopic (R0 or R1) resection for ductal adenocarcinoma of the pancreas with histological confirmation and with no evidence of malignant ascites, peritoneal metastasis, or spread to the liver or other distant abdominal or extra-abdominal organs. The type and extent of resection were determined using an established international classification.[16] Patients had to be fully recovered from the operation, with a World Health Organization performance score of 2 or lower and a life expectancy of more than 3 months. Patients with previous use of neoadjuvant chemotherapy or other concomitant chemotherapy and with pancreatic lymphoma, macroscopically remaining tumor (R2 resection), or TNM stage IVb disease were excluded.

### Randomization
Patients were randomly assigned to each treatment group on a 1:1 basis according to a computer-generated variable-size blocked randomization method. Patients were stratified at randomization by country and resection margin status (R0 vs R1).

### Chemotherapy
Folinic acid (20 mg/m$^2$) was given as an intravenous bolus followed by intravenous bolus fluorouracil (425 mg/m$^2$) given on 5 consecutive days every 28 days for 6 cycles (24 weeks). Gemcitabine (lyophilized powder diluted in normal saline) was given as an intravenous infusion over 30 minutes (1000 mg/m$^2$), administered once a week for 3 out of every 4 weeks (1 cycle) for 6 cycles (24 weeks). Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria for Adverse Events (version 2), with a clearly defined protocol for modifications and delays.

Quality of life was assessed using the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 (version 2) and ESPAC-32 patient questionnaires at baseline and at 3 and 6 months and yearly until 5 years.[17]

### Statistical Analysis
The trial was designed to test the primary hypothesis, ie, that overall length of survival does not differ between that achieved with adjuvant fluorouracil plus folinic acid and that achieved with gemcitabine. Secondary end points were progression-free survival, toxicity, and quality of life. Power calculations were based on expected 2-year survival rates. The ESPAC-1 trial had shown that 2-year survival with fluorouracil plus folinic acid was in the order of 40% to 45%.[6,12] ESPAC-3 was powered to detect a clinically meaningful increase in survival of 10% with gemcitabine. Recruiting 515 patients (275 deaths) in each treatment group would allow 10% differences in 2-year survival to be detected using a 2-sided $\alpha$ = .05 level of significance with at least 90% power.

Overall survival was measured from the date of resection to date of death from any cause. Patients remaining alive were censored at the date last seen alive. Progression-free survival was measured from date of resection to date of death from any cause or date of local tumor recurrence or metastasis. Patients remaining alive and progression-

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free were censored at the date last seen alive. Survival estimates were calculated using the Kaplan-Meier method[18] and compared using the unweighted Mantel-Haenszel version of the log-rank test.[19] Median, 12-month, and 24-month survival estimates are presented with 95% confidence intervals (CIs).

The hazard ratio (HR) of the treatment effect is presented for gemcitabine compared with that for fluorouracil plus folinic acid. Hazard ratios of the treatment effect within stratification subgroups at randomization are estimated (without significance testing) with tests of heterogeneity to determine if treatment effects differ across subgroups. The treatment effect was adjusted by stratification factors at randomization (country and resection margin status) and other identified prognostic factors in the multivariate setting using Cox proportional hazards modeling[30] incorporating a random effect into the hazard function for country effect. Factors with a log-rank significance of $P < .10$ were explored further in the multivariate setting using backward selection techniques. Classification variables were used for ordinal variables with more than 2 categories. The functional form of the relationship between continuous factors and log-hazard (specifically age, tumor size, and postoperative carbohydrate antigen 19-9 [CA19-9] level) was assessed, and factors were included in the multivariate models with a nonlinear transformation if appropriate.[21] The assumption of proportional hazards was assessed and confirmed by including a time-dependent covariate.

The number of patients receiving treatment and the percentage of protocol dose of chemotherapy and the range of total doses received was calculated. The number of patients experiencing at least 1 high-grade toxic episode (grade 3/4) of each toxicity type or serious adverse event is reported as a percentage of the total number of patients randomized within each treatment group. Proportions were compared using the Fisher exact test with the significance level set at $P < .005$ and
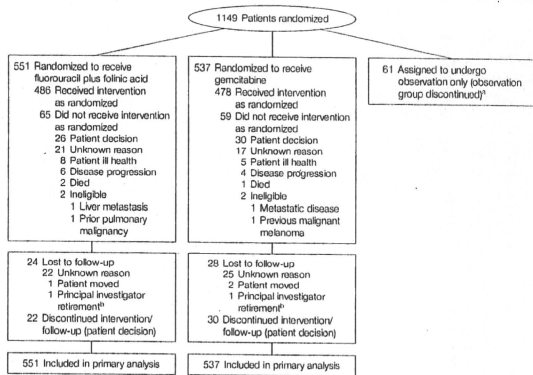
with Bonferroni adjustment to account for multiple testing.

Quality-of-life domain scores were calculated according to the EORTC QLQ-C30 scoring manual and linearly transformed to produce a standardized score ranging from 0 to 100. Higher scores for the functional and global health scales indicated better quality of life, whereas higher scores for the symptom scales and items indicated poorer quality of life. Standardized area under the curve (AUC) scores[17] are average observed symptomatic and functional quality-of-life scores per month within a 12-month duration from surgery, calculated from the linearly transformed scores and compared across treatments using the Mann-Whitney nonparametric test.

All statistical analyses were carried out using SAS version 9.1 (SAS Institute Inc, Cary, North Carolina) and R version 2.7.2 (R Project for Statistical Computing; http://www.r-project.org) on an intention-to-treat basis, retaining patients in their randomized treatment groups and including proto-

col violators and ineligible patients. A 2-sided significance level of $P < .05$ was used throughout.

## RESULTS

The last of the 1088 patients recruited was randomized on January 8, 2007. The database was locked on March 18, 2009.

### Patient Characteristics

Five hundred fifty-one patients were randomized to receive fluorouracil plus folinic acid, and 537 were randomized to receive gemcitabine (FIGURE 1). Four ineligible patients were reported (2 in each group) and have been included in the analysis on an intention-to-treat basis. The clinical characteristics of patients and surgical and pathological details are shown in TABLE 1.

### Treatment

Four hundred eighty-six patients (88%) received 2326 cycles of fluorouracil plus folinic acid and 478 (89%) received 2464 cycles of gemcitabine. Sixty-five patients (12%) in the fluorouracil plus

**Figure 1.** ESPAC-3 Study Flow



ESPAC indicates European Study Group for Pancreatic Cancer.
[a] Discontinued in June 2003 owing to statistical evidence for survival benefit attributable to adjuvant chemotherapy.
[b] Principal investigator at research site retired from practice with no replacement.

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**Table 1.** Patient Characteristics at Randomization

| Characteristic | Fluorouracil + Folinic Acid (n=551) | Gemcitabine (n=537) | Total (N=1088) |
|---|---|---|---|
| Sex | | | |
| Men | 301 (55) | 297 (55) | 598 (55) |
| Women | 250 (45) | 240 (45) | 490 (45) |
| Age, y | | | |
| Median (IQR) | 63 (56-70) | 63 (56-69) | 63 (56-69) |
| Range | 34-85 | 31-81 | 31-85 |
| Performance score | | | |
| 0 | 201 (36) | 170 (32) | 371 (34) |
| 1 | 286 (52) | 303 (56) | 589 (54) |
| 2 | 64 (12) | 64 (12) | 128 (12) |
| Smoking status | | | |
| Never | 207 (43) | 189 (40) | 396 (41) |
| Past | 192 (39) | 207 (44) | 399 (42) |
| Present | 87 (18) | 78 (16) | 165 (17) |
| Missing | 65 | 63 | 128 |
| Concurrent conditions | | | |
| None | 240 (46) | 263 (52) | 503 (49) |
| Yes | 277 (54) | 240 (48) | 517 (51) |
| Missing | 34 | 34 | 68 |
| Diabetes | | | |
| No | 388 (75) | 375 (75) | 763 (76) |
| Non–insulin-dependent | 54 (11) | 51 (10) | 105 (10) |
| Insulin-dependent | 72 (14) | 73 (15) | 145 (14) |
| Missing | 37 | 38 | 75 |
| Postoperative CA19-9 level | | | |
| No. | 394 | 373 | 767 |
| Median (IQR), kU/L | 26 (10-65) | 22 (9-62) | 24 (10-63) |
| Time from surgery to randomization, median (IQR), d | 45 (29-57) | 45 (30-57) | 45 (29-57) |
| Hospital stay | | | |
| No. | 494 | 478 | 972 |
| Median (IQR), d | 14 (10-20) | 14 (10-20) | 14 (10-20) |
| Resection margins | | | |
| Negative | 356 (65) | 348 (65) | 704 (65) |
| Positive | 195 (35) | 189 (35) | 384 (35) |
| Tumor grade | | | |
| Well differentiated | 81 (15) | 66 (13) | 147 (14) |
| Moderately differentiated | 327 (60) | 336 (63) | 663 (62) |
| Poorly differentiated | 135 (25) | 125 (24) | 260 (24) |
| Undifferentiated | 2 (0) | 2 (0) | 4 (0) |
| Lymph nodes | | | |
| Negative | 162 (30) | 145 (27) | 307 (28) |
| Positive | 387 (70) | 391 (73) | 778 (72) |
| Maximum tumor size | | | |
| No. | 526 | 507 | 1033 |
| Median (IQR), mm | 30 (23-40) | 30 (24-40) | 30 (23-40) |
| Tumor stage[a] | | | |
| I | 58 (11) | 46 (9) | 104 (10) |
| II | 154 (28) | 144 (27) | 298 (28) |
| III | 303 (56) | 319 (61) | 622 (58) |
| IVa | 26 (5) | 16 (3) | 42 (4) |
| Surgery | | | |
| Whipple resection | 290 (56) | 299 (59) | 589 (58) |
| Total pancreatectomy | 28 (5) | 15 (3) | 43 (4) |
| Pylorus-preserving resection | 162 (31) | 150 (30) | 312 (30) |
| Distal pancreatectomy | 40 (8) | 40 (8) | 80 (8) |

*(continued)*

folinic acid group and 59 (11%) in the gemcitabine group did not start treatment. Three hundred one patients (55%) in the fluorouracil plus folinic acid group and 323 (60%) in the gemcitabine group received all 6 cycles of treatment. Median time from randomization to the start of chemotherapy was 10 (interquartile range [IQR], 5-18) days for the fluorouracil plus folinic acid group and 8 (IQR, 5-14) days for the gemcitabine group. Median time receiving chemotherapy was 4.7 (IQR, 3.1-5.0) months for the fluorouracil plus folinic acid group and 5.1 (IQR, 4.0-5.3) months for the gemcitabine group. Median dose intensity was 79% (range, 3%-141%) of the planned protocol for the fluorouracil plus folinic acid group and 89% (range, 6%-122%) for the gemcitabine group.

**Overall Survival**

Seven hundred fifty-three patients (69%) had died at the time of analysis (388 [70%] in the fluorouracil plus folinic acid group and 365 [68%] in the gemcitabine group). Median length of follow-up of 335 living patients was 34.2 (IQR, 27.1-43.4; range, 0.4-86.3) months, equal across treatment groups. Overall, 282 of patients remaining alive (84%) had undergone follow-up for more than 2 years. Median survival was estimated as 23.2 months (95% CI, 21.7-24.9), with 12-month and 24-month rates estimated as 79.3% (95% CI, 76.9%-81.8%) and 48.6% (95% CI, 45.6%-51.6%), respectively. Median survival for patients treated with fluorouracil plus folinic acid was 23.0 (95% CI, 21.1-25.0) months and for patients treated with gemcitabine was 23.6 (95% CI, 21.4-26.4) months (FIGURE 2).

Survival estimates at 12 and 24 months were 78.5% (95% CI, 75.0%-82.0%) and 48.1% (95% CI, 43.8%-52.4%), respectively, for the fluorouracil plus folinic acid group and 80.1% (95% CI, 76.7%-83.6%) and 49.1% (95% CI, 44.8%-53.4%) for the gemcitabine group. Log-rank analysis revealed no statistically significant difference in survival estimates between

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