BSC alone is ~2.5–4.5 months and palliative chemotherapy should be considered as a treatment option. A systematic review of chemotherapy studies for advanced BTC published in 2007 identified 104 studies since 1985. The largest study reported 65 patients; there was one phase III study (closed early due to poor recruitment) and two randomised phase II studies [11]. No standard regimen was identified although the most active regimens appeared to be those including gemcitabine alone, gemcitabine with a platinum agent or 5-FU with a platinum agent.

The Advanced biliary tract cancer (ABC)-02 study (Clinical Trials.gov number: NCT00262769) was a UK-wide phase III study, carried out under the auspices of the National Cancer Research Network, comparing doublet-chemotherapy (CisGem, cisplatin 25 mg/m^2 and gemcitabine 1000 mg/m^2, each on days 1 and 8 of a 21-day regimen) versus gemcitabine monotherapy (Gem, gemcitabine 1000 mg/m^2 on days 1, 8 and 15 of a 28-day regimen). It built on a randomised phase II study (ABC-01, at the time the largest global study with 86 patients) [12], which had demonstrated an improvement in 6-month progression-free survival from 47.7% to 57.1%. ABC-01 was then extended to the phase III ABC-02 study using an identical protocol but recruiting an additional 324 patients for a total of 410 patients. This extension would provide statistical power for an overall survival (OS) analysis. This study demonstrated a statistically improved OS in favour of the combination arm [median OS 11.7 versus 8.1 months, hazard ratio (HR) 0.64; 95% confidence interval (CI) 0.52–0.80, $P < 0.001$] with an improved progression-free survival [PFS, median 8.0 versus 5.0 months, HR = 0.63, 95% CI 0.51–0.77, $P < 0.001$) and an acceptable toxicity profile, [13] thus setting a reference regimen for patients with advanced BTC.

In parallel, the biliary tract (BT) 22 study (ClinicalTrials.gov number NCT00380588) was developed in Japan in order to replicate the ABC-01 data using an identical regimen to the ABC studies. Compared with Gem, patients who received CisGem had a better 1-year survival (the primary end point, 39.0% versus 31.0%); median OS (11.2 versus 7.7 months); median PFS (5.8 versus 3.7 months) and radiological response rate (19.5% versus 11.9%). The hazard ratio between the treatment arms was 0.69 (95% CI 0.42–1.13) for OS and 0.66 (95% CI 0.41–1.05) for PFS in favour of CisGem [14].

The meta-analysis reported here represents a pre-planned international collaboration between UK and Japanese investigators in order to achieve greater statistical power in the evaluation of the treatment effect.

patients and methods

The primary objective of this meta-analysis is to evaluate the effect of cisplatin and gemcitabine (CisGem) versus gemcitabine alone (Gem), with enhanced patient numbers by combining patient-level data from the ABC-01/ABC-02 and BT22 studies (Table 1). In addition, we sought to explore the relative treatment effect across both studies given the inherent differences between the study populations.

Each of the studies was carried out with Ethics Committee and other requisite approvals/notifications (governed by the country of each study sponsor); all patients were enrolled after giving informed consent to participate and the studies were conducted in accordance with the Declaration of Helsinki.

Gemcitabine was provided for the investigators in both studies by Lilly Oncology or Eli Lilly Japan, as appropriate. ABC-02 was carried out as an investigator-initiated academic study; Lilly Oncology was not involved in the accrual or analysis of the data, or the preparation of the manuscript. Data from ABC-02 were held by the study sponsor, University College London Clinical Trials Unit (UCL CTU). BT22 was originally a Lilly-sponsored trial although additional data collection for OS and PFS was made as an investigator-initiated study and supported by the Ministry of Health, Labour and Welfare (MHLW), Health Labour Sciences Research Grant; data were collected by the investigators and released following publication of BT22 to UCL CTU under a study-specific agreement for the sole purposes of this meta-analysis.

Cox proportional hazards model was used to estimate the effect of treatment on OS and PFS, providing a HR for CisGem versus Gem. The treatment effect was examined for each of the subgroups for pre-specified baseline factors, as well as age, sex and trial. A test for interaction with treatment was assessed for each set of subgroups.

OS was estimated from date of entry to the trial to date of death, or date last seen alive; PFS was estimated from date of entry to the trial until date of

Table 2. Baseline characteristics among ABC-02 and BT22 trial patients, by treatment

Baseline factor	No. (%)	
	Gemcitabine + cisplatin ($N = 245$)	Gemcitabine alone ($N = 248$)
Sex		
Female	131 (53)	129 (52)
Male	114 (47)	119 (48)
Age (years): median (range)	64 (32–81)	64 (23–84)
Disease status		
Locally advanced	60 (24)	57 (23)
Metastatic	174 (71)	181 (73)
Not stated	11 (4)	10 (4)
Primary tumour site[a]		
Intra-hepatic	51 (21)	57 (23)
Extra-hepatic	76 (31)	73 (29)
Gallbladder	88 (36)	93 (38)
Ampulla	13 (5)	11 (4)
Not stated	17 (7)	14 (6)
Histology		
Adenocarcinoma	225 (92)	232 (94)
Carcinoma unspecified	17 (7)	12 (5)
Adenosquamous carcinoma	2 (1)	3 (1)
Squamous-cell carcinoma	0 (0)	1 (<1)
Carcinosarcoma	1 (<1)	0 (0)
ECOG performance status		
0	100 (41)	92 (37)
1	118 (48)	131 (53)
2	27 (11)	25 (10)
Prior therapy		
No	80 (33)	78 (31)
Yes	165 (67)	170 (69)

[a]Hilar patients from ABC-02 are included in the extra-hepatic group.

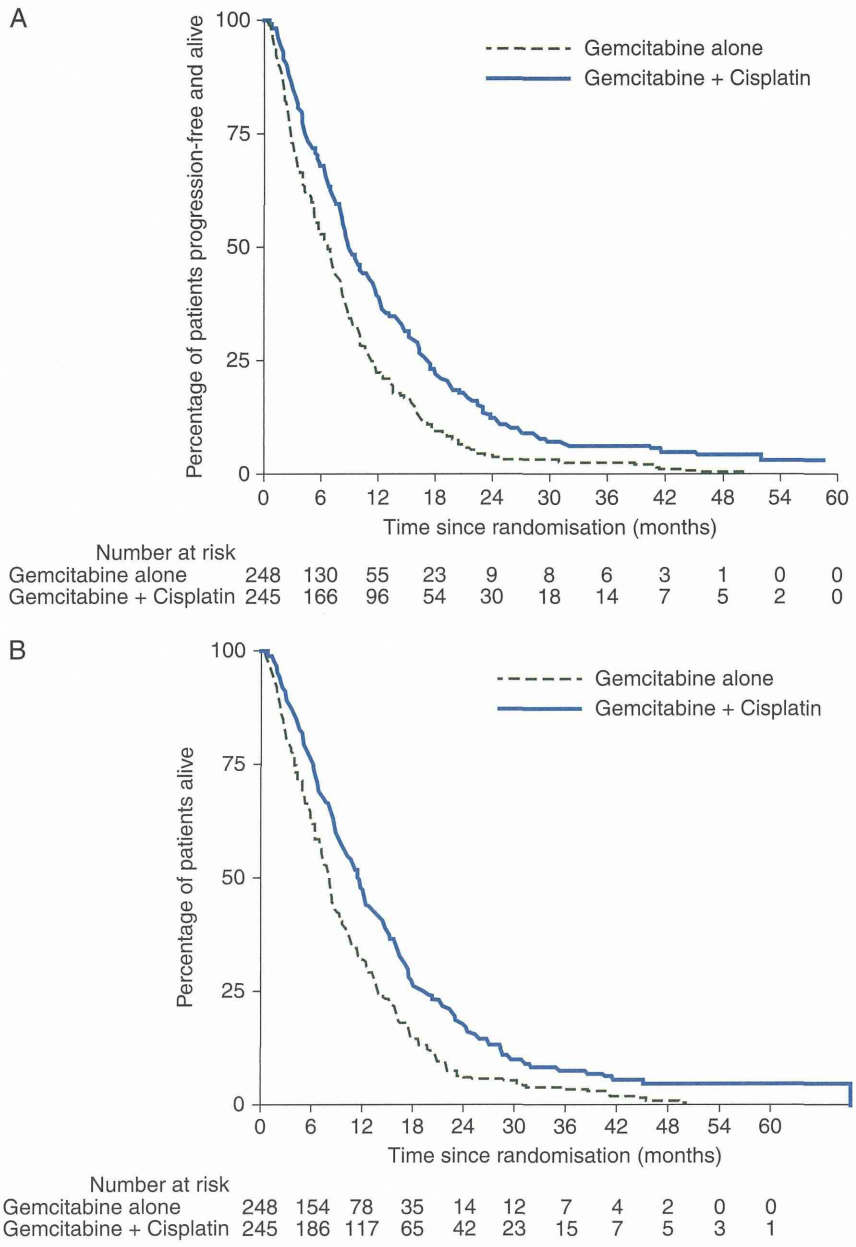

Figure 1. (A) Kaplan–Meier curve for progression-free survival, by treatment. Hazard ratio = 0.64 (95% CI 0.53–0.76), $P < 0.001$. (B) Kaplan–Meier curve for overall survival, by treatment. Hazard ratio = 0.65 (95% CI 0.54–0.78), $P < 0.001$.

progression or date of death, or date last seen alive for those patients without either event.

results

A total of 493 patients, median age 64 years (range 23–84 years) with approximately equal sex distribution, were randomised (ABC-02 study $n = 410$; BT22 study $n = 83$) to receive either CisGem [$N = 245$: ABC-02 ($n = 204$); BT22 ($n = 41$)] or Gem [$N = 248$: ABC-02 ($n = 206$); BT22 ($n = 42$)] (Table 2). Three-quarters of the patients had metastatic disease; 89% of patients had a good performance status (PS, 0-1) (patients with PS of 2 were eligible for ABC-02, but not BT22); and the histology was of adenocarcinoma type in 93% with a small number of patients with alternative histologies (Table 2). Sixty-eight percent of patients had prior therapy, primarily in the form of biliary stenting; a total of 109 patients (22%) had undergone prior surgery with curative intent and subsequently relapsed; prior systemic chemotherapy for advanced disease was not allowed.

These data had slightly longer follow-up in both trials (median follow-up in ABC-02: 9.2 months; BT22: 9.0 months), compared with the published papers [13, 14]. When compared with gemcitabine monotherapy, the combination of cisplatin and gemcitabine was associated with an improved PFS (median 8.8 versus 6.7 months; HR = 0.64 (95% CI 0.53–0.76), $P < 0.001$) and OS (median 11.6 versus 8.0 months; HR = 0.65 (95% CI 0.54–0.78), $P < 0.001$), Figure 1a and b, respectively. Thus, the use of combination chemotherapy reduces the risk of progression or death (defined by PFS event) by 36%; and risk of death by 35%, compared with gemcitabine monotherapy.

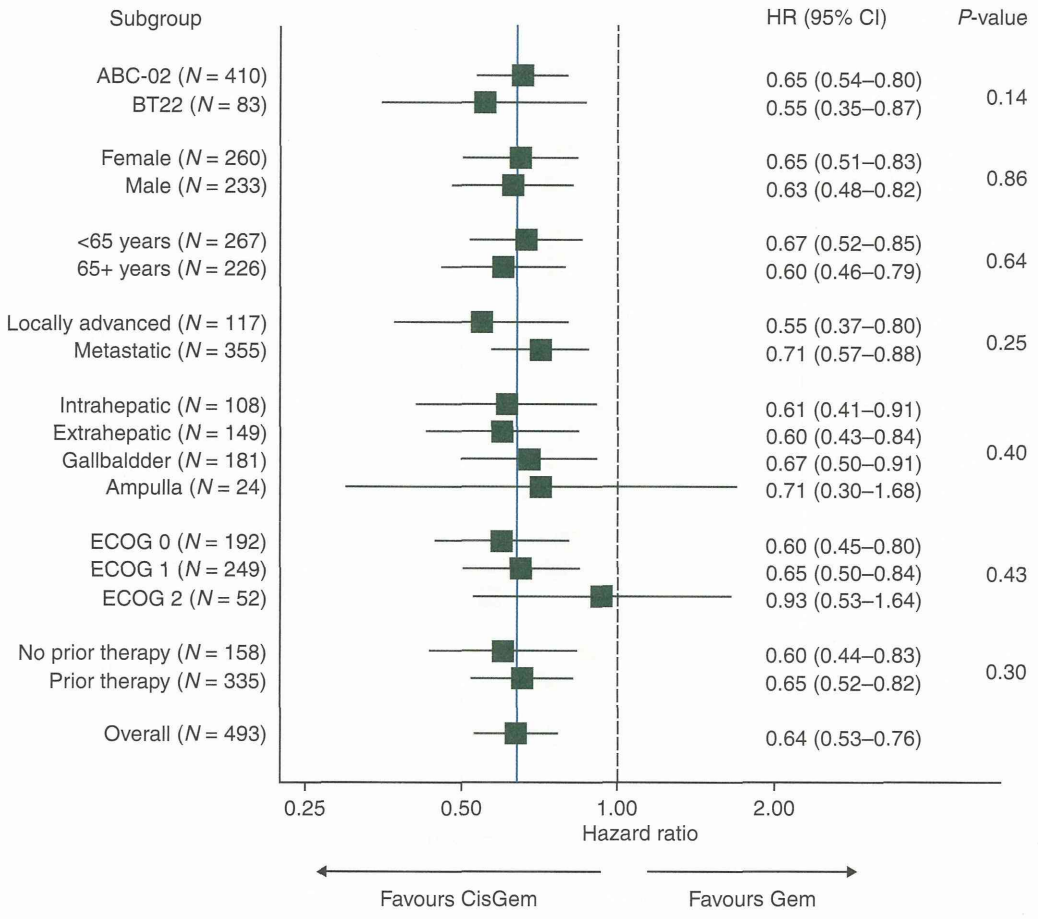

Figure 2. Progression-free survival, among ABC-02 and BT22 trials, by subgroups. The hazard ratio (95% confidence interval) for the treatment effect (CisGem versus Gem alone) is provided for each subgroup, per factor and the corresponding *P*-value for the test of interaction between treatment and factor. The forest plot excludes patients with unstated disease status and tumour site subgroup levels.

Exploratory subgroup analysis suggests that all patients benefit from CisGem versus Gem with respect to sex; age (<65 and ≥65 years), stage of disease (locally advanced and metastatic); site of primary tumour (intra-hepatic, extra-hepatic cholangiocarcinoma and gallbladder cancer); performance score (PS 0 and 1) and use of prior therapy (Figures 2 and 3). The widest confidence intervals are seen in patients with ampullary tumours and those with PS 2 due to the small size of each cohort ($n = 24$ and $n = 52$, respectively). When limited to patients with PS 0-1 only ($n = 441$), the HR for PFS is 0.61 (95% CI 0.51–0.74), $P < 0.001$ and for OS HR = 0.64 (95% CI 0.53–0.77), $P < 0.001$.

There is no evidence for a difference in treatment effects between any of the subgroups for PFS or OS (Figures 2 and 3). The treatment effect is remarkably similar between the two studies (BT22 versus ABC-02) with respect to OS (Figure 3; HR = 0.65 for both trials) and PFS (Figure 2) [test for heterogeneity for OS: $P = 0.90$; PFS: $P = 0.14$].

A total of 109 patients had surgery before trial entry; there is no evidence of an interaction between prior surgery status and treatment effect for OS and PFS ($P = 0.52$ and $P = 0.26$, respectively).

discussion

It was previously believed that the incidence of BTC was too low for prospective, adequately powered clinical studies to be carried out. This meta-analysis, achieved by international collaboration, combines individual patient-level data from two prospective randomized, controlled trials in pathologically proven, advanced BTC using the same treatment comparisons [the UK ABC-02 phase III study ($n = 410$) and Japanese BT22 randomised phase II study ($n = 84$)] and thus represents the largest prospectively evaluated patient pool with close to 500 patients in total. This meta-analysis demonstrates a significant improvement in PFS and OS in favour of cisplatin and gemcitabine doublet chemotherapy over gemcitabine monotherapy, of the order of a 35% reduction in the risk of outcome.

There is a striking consistency between the treatment effect observed between the ABC-02 and BT22 studies (both HRs = 0.65) with respect to OS (Figure 3) with near-reproducible median survival in the combination arms (11.7 and 11.1 months, respectively). The similarity is less marked for PFS (HR 0.65 and 0.55), with median PFS of 9.7 and 6.5 months in each of the combination arms of ABC-02 and BT22, respectively. This is likely to be due to differences in protocol-driven assessments; specifically frequency of radiological tumour reassessment (6-weekly in BT22 and 12-weekly in ABC-02) [15].

This meta-analysis did not include an assessment of toxicity due to the different schedules for safety assessment between the protocols (specifically, BT22 included assessment of complete blood count and biochemistry on the rest week of treatment

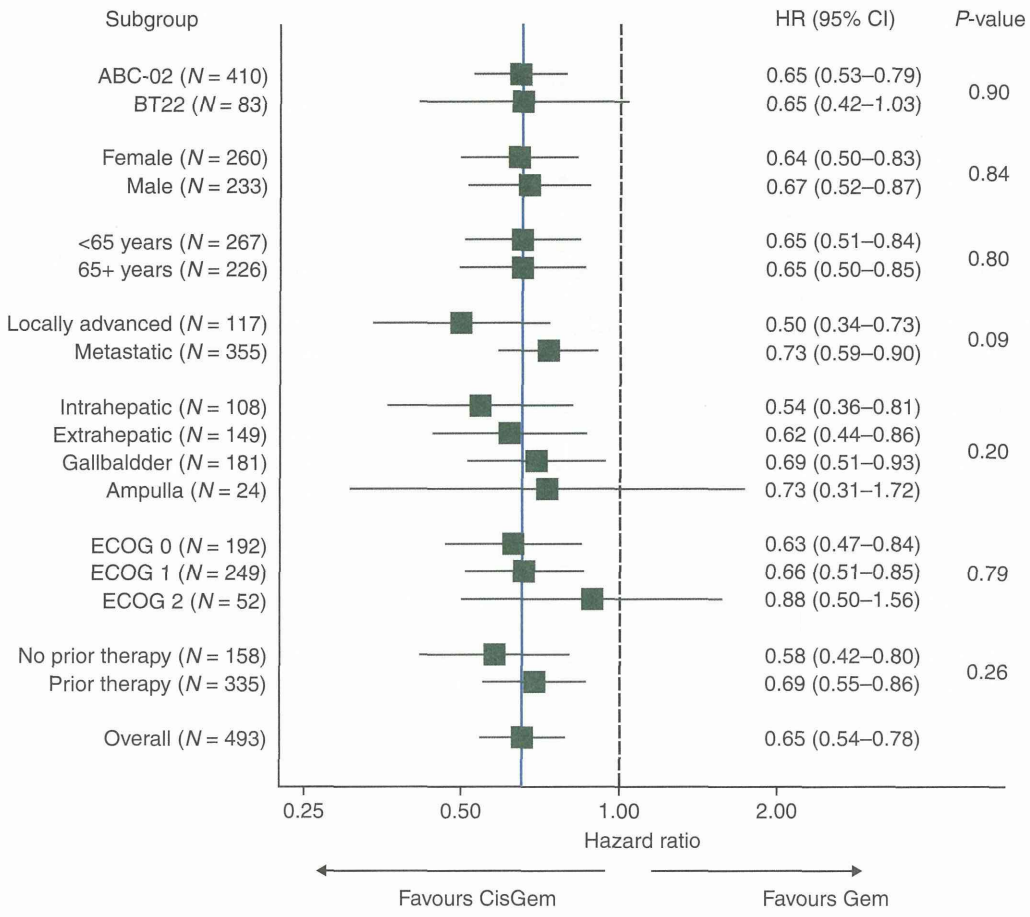

Figure 3. Overall survival, among ABC-02 and BT22 trials, by subgroups. The hazard ratio (95% CI) for the treatment effect (CisGem versus Gem alone) is provided for each subgroup, per factor and the corresponding *P*-value for the test of interaction between treatment and factor. The forest plot excludes the undefined disease status and tumour site subgroup levels.

(not required for ABC-02) which may explain the increased haematological toxicity reported in this study; [15] however, these regimens are well established and toxicities are detailed in the individual study publications [13, 14].

Patients with a good PS (0-1) appear to derive greater benefit from combination chemotherapy (HR for PFS and OS are 0.61 and 0.64, respectively). It is therefore appropriate for future studies using this combination chemotherapy to limit inclusion to PS 0-1 patients. PS2 patients were only included in the ABC-02 study and the HR for OS for this group was 0.88, 95% CI 0.50–1.56; thus, in the absence of studies specifically addressing therapy for PS2 patients, it may be preferable to consider gemcitabine monotherapy for this group given the very poor survival with BSC alone [9, 10].

With the exception of ampullary tumours combination chemotherapy resulted in statistically significantly favourable PFS and OS for all other tumour-location subgroups (intrahepatic cholangiocarcinoma, extra-hepatic cholangiocarcinoma and gallbladder cancer). Although a reduced risk of 25%–30% was seen in the ampullary group, the small numbers did not permit a statistically meaningful result. As these cancers are uncommon, it may be necessary for another meta-analysis to provide the statistical power required for a robust assessment of this tumour subtype in future studies.

The remit of this meta-analysis is limited to the effect of first-line chemotherapy for patients with advanced BTC. The use of subsequent chemotherapy can confound the survival analysis. Only 18% of patients in the ABC-02 study went on to receive second-line chemotherapy, primarily due to there being no UK-recognised regimen in this setting. In contrast, 76% of patients in BT22 went on to receive second-line chemotherapy on disease progression in Japan where the oral fluoropyrimidine, S1, is a licensed treatment option for these patients. Despite this disparity, the survival in the combined arms was very similar as already discussed, accepting the inherent limitations of cross-study comparisons. There are no randomised phase III data that second-line chemotherapy improves survival for patients who have previously been treated with cisplatin and gemcitabine; specifically, no phase III studies have ever been carried out. A recent large retrospective single-centre series suggests that second-line chemotherapy (after a heterogeneous group of first-line regimens) is feasible in ~25% of patients; [16] moreover, after cisplatin and gemcitabine first-line chemotherapy, we have shown that patients who do go on to receive chemotherapy may derive additional benefit (median survival from start of second-line treatment: 8.1 months, median survival from start of first-line chemotherapy: 19.5 months) [17]. However, such analysis is highly subject to selection bias and prospective studies are

urgently needed to determine the benefit (if any) in terms of survival, impact on quality of life and cost-effectiveness of second-line chemotherapy.

This meta-analysis for efficacy, together with a recently published cost-effectiveness analysis [18] has strengthened the rationale for recommending cisplatin and gemcitabine as a reference regimen for development of further therapies across international patient populations with advanced BTC.

acknowledgements

We thank all participating patients and their families without whom these clinical studies would not have been possible. This has been an international collaborative effort on behalf of the investigators detailed below.

ABC-02 study recruiting sites (UK) and investigators are as follows: Aberdeen Royal Infirmary—M. Nicholson; Addenbrooke's Hospital—P. Corrie; Belfast City Hospital—M. Eatock; Bristol Royal Infirmary—S. Falk; Cheltenham General Hospital—S. Elyan; Christie Hospital—J. Valle (co-chief investigator); Cookridge Hospital—A. Anthony; Cumberland Infirmary—J. Nicoll; Derbyshire Royal Infirmary—R. Kulkarni; Dorset Cancer Centre—R. Osbourne; Glan Clwyd Hospital—A. Garcia Alonso; Hammersmith Hospital—H. Wasan (co-chief investigator); Maidstone Hospital—J. Waters; Mount Vernon Hospital—M. Harrison; Ninewells Hospital—D. Adamson; North Hampshire Hospital—C. Rees; North Middlesex Hospital—J. Bridgewater (co-chief investigator); Nottingham University Hospital—S. Madhusudan; Peterborough Hospital—K. McAdam; Princess Alexandra Hospital—J. Bridgewater (co-chief investigator); Princess Royal Hospital—A. Maraveyas; Queen Elizabeth Hospital Birmingham—D. Palmer; Royal Bourne-mouth Hospital—T. Hickish; Royal Free Hospital—T. Meyer; Royal Marsden Hospital—D. Cunningham; Royal South Hants Hospital—T. Iveson; Royal Surrey County Hospital—G. Middleton; St. Bartholomew's Hospital—S. Slater; St. George's Hospital—F. Lofts; St. Mary's Hospital Portsmouth—C. Archer; Salisbury Hospital—T. Iveson; Southampton General Hospital—T. Iveson; University College Hospital—J. Bridgewater (co-chief investigator); Velindre Cancer Centre—S. Mukherjee; Weston Park Hospital—J. Wadsley; Wrexham Maelor Hospital—S. Gollins.

BT22 study recruiting sites (Japan) and investigators are as follows: T. Okusaka—Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, Tokyo; K. Nakachi—Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, Kashiwa; A. Fukutomi—Division of Gastrointestinal Oncology, Shizuoka Cancer Center, Shizuoka; N. Mizuno—Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya; S. Ohkawa—Division of Hepatobiliary and Pancreatic Medical Oncology, Kanagawa Cancer Center, Yokohama; A. Funakoshi—Division of Gastroenterology, Kyushu Cancer Center, Fukuoka; M. Nagino—Division of Surgical Oncology, Nagoya University Graduate School of Medicine, Nagoya; S. Kondo—Department of Surgical Oncology, Hokkaido University Graduate School of Medicine, Sapporo; J. Furuse—Department of Internal Medicine, Medical Oncology, Kyorin University School of Medicine, Tokyo; M. Miyazaki—Department of General Surgery, Chiba University Graduate School of Medicine, Chiba; and Y. Nimura—Aichi Cancer Center, Nagoya.

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disclosure

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Pharmacogenetic predictors of severe peripheral neuropathy in colon cancer patients treated with oxaliplatin-based adjuvant chemotherapy: a GEMCAD group study

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Background: Oxaliplatin-based chemotherapy (CT), widely used as adjuvant therapy for stage III and selected high-risk stage II colon cancer (CC) patients, is often associated with cumulative peripheral neuropathy. Our aim is to identify single-nucleotide polymorphisms (SNPs) in genes involved in oxaliplatin metabolism, DNA repair mechanisms, cell cycle control, detoxification or excretion pathways to predict severe (grade 2–3) oxaliplatin-induced peripheral neuropathy (OXPN) among CC patients treated with oxaliplatin and fluoropyrimidine-based adjuvant CT.

Patients and methods: Genomic DNA was extracted from formalin-fixed-paraffin-embedded peritumoral samples from 206 high-risk stage II and stage III CC patients receiving oxaliplatin-based adjuvant CT from January 2004 to December 2009. Genotyping was carried out for 34 SNPs in 15 genes using MassARRAY (SEQUENOM) technology. A total of 181 stage II–III CC patients treated with the same CT regimens were enrolled as a validation set.

Results: The rs2230641 cyclin H (CCNH) rs2230641 C/C [odd ratio (OR) = 5.03, 95% confidence interval (CI) 1.061–2.41, $P = 0.042$] and the ATP-binding cassette subfamily G, member 2 (ABCG2) rs3114018 A/A genotypes (OR = 2.67; 95% CI 0.95–4.41; $P = 0.059$) were associated with a higher risk of severe OXPN. In addition, patients harboring the combination of CCNH C/C and/or the ABCG2 rs3114018 A/A genotypes had a higher risk of grade 2–3 OXPN than those with the CCNH any T and ABCG2 any C genotypes (37.73% versus 19.42%; OR = 2.46; 95% CI 1.19–5.07; $P = 0.014$) in the logistic regression analysis using age, gender, adjuvant CT regimen and cumulative dose of oxaliplatin as covariates. The ability to predict severe OXPN of this combined analysis was independently validated in the second cohort (58% versus 33.33%; OR = 2.99; 95% CI 1.45–6.13; $P = 0.002$).

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Fibroblast Growth Factor Receptor 2 Tyrosine Kinase Fusions Define a Unique Molecular Subtype of Cholangiocarcinoma

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Cholangiocarcinoma is an intractable cancer, with limited therapeutic options, in which the molecular mechanisms underlying tumor development remain poorly understood. Identification of a novel driver oncogene and applying it to targeted therapies for molecularly defined cancers might lead to improvements in the outcome of patients. We performed massively parallel whole transcriptome sequencing in eight specimens from cholangiocarcinoma patients without *KRAS/BRAF/ROS1* alterations and identified two fusion kinase genes, *FGFR2-AHCYL1* and *FGFR2-BICC1*. In reverse-transcriptase polymerase chain reaction (RT-PCR) screening, the *FGFR2* fusion was detected in nine patients with cholangiocarcinoma (9/102), exclusively in the intrahepatic subtype (9/66, 13.6%), rarely in colorectal (1/149) and hepatocellular carcinoma (1/96), and none in gastric cancer (0/212). The rearrangements were mutually exclusive with *KRAS/BRAF* mutations. Expression of the fusion kinases in NIH3T3 cells activated MAPK and conferred anchorage-independent growth and *in vivo* tumorigenesis of subcutaneous transplanted cells in immune-compromised mice. This transforming ability was attributable to its kinase activity. Treatment with the fibroblast growth factor receptor (FGFR) kinase inhibitors BGJ398 and PD173074 effectively suppressed transformation. *Conclusion: FGFR2* fusions occur in 13.6% of intrahepatic cholangiocarcinoma. The expression pattern of these fusions in association with sensitivity to FGFR inhibitors warrant a new molecular classification of cholangiocarcinoma and suggest a new therapeutic approach to the disease. (HEPATOLOGY 2013;00:000-000)

Cholangiocarcinoma (CC) is a highly malignant invasive carcinoma that arises through malignant transformation of cholangiocytes.[1] It is an intractable tumor with poor prognosis, whose incidence and mortality rates are high in East Asia and have been rapidly increasing worldwide.[1,2] CC can be subdivided into intrahepatic (ICC) and extrahepatic (ECC) types, which show distinct etiological and clinical features.[2] ICC is the second most common primary hepatic malignancy after hepatocellular carcinoma, and is associated with hepatitis virus infection. Somatic mutations of *KRAS* and *BRAF* are the most common genetic alterations in CC.[3,4] Surgical resection is the only curative treatment for CC, and no standard chemotherapy regimens have been established for inoperative cases or those showing recurrence after surgical resection.[5,6]

Abbreviations: CC, cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; FGFR, fibroblast growth factor receptor; FISH, fluorescent in situ hybridization; ICC, intrahepatic cholangiocarcinoma; TKI, tyrosine kinase inhibitor.

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**These authors contributed equally to this work.*

A better understanding of the molecular basis of cancer would help develop targeted therapeutic agents against druggable genetic aberrations identified in cancer genomes.[7,8] Tyrosine kinase inhibitors (TKIs) that target anaplastic lymphoma kinase (ALK) are particularly effective in the treatment of a distinct subset of lung adenocarcinoma carrying *ALK* fusions.[9] *FIG-ROS1*, the first identified targetable fusion kinase in CC, has so far been reported in two patients.[10] Very recently, a novel kinase fusion, *FGFR2-BICC1*, was detected in two CC cases.[11] Thus, only a few cases harboring targetable fusion kinase genes have been reported, and the clinical characteristics of fusion-positive CC cases have not yet been described.

In the present study, we identified fibroblast growth factor receptor 2 (*FGFR2*) rearrangements including a novel *FGFR2-AHCYL1* fusion using whole transcriptome high-throughput sequencing of tumor specimens, and determined the prevalence of *FGFR2* rearrangements in CC. Our data indicate that *FGFR2*-fusions arise exclusively in ICC. *In vitro* studies suggest that *FGFR2* fusion kinase is a promising candidate for targeted therapy in CC.

Materials and Methods

Clinical Samples. Clinical specimens of cholangiocarcinoma, gastric cancer, hepatocellular carcinoma, and colorectal cancer were provided by the National Cancer Center Biobank, Japan. Total RNA was extracted from grossly dissected, snap-frozen tissue using RNAspin (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions, and RNA quality was examined using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The study protocol was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

Analysis of Whole Transcriptome Sequence Data. Complementary DNA (cDNA) libraries composed of 150-200 bp inserts were prepared from 2 μg of total RNA using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA). The libraries were subjected to paired-end sequencing of 50-100 bp fragments on the HiSeq2000 instrument (Illumina) according to the manufacturer's instructions. Paired-end reads were mapped to known RNA sequences in the RefSeq, Ensembl, and LincRNA databases using the Bowtie program (v. 0.12.5) as basically described previously.[12] The detailed algorithm for fusion transcript detection is described in the Supporting Methods.

RT-PCR and Quantitative Real-Time PCR. Total RNA was reverse-transcribed to cDNA using SuperScript III (Life Technologies, Carlsbad, CA). The cDNA was subjected to PCR amplification using Ex-Taq (Takara Bio, Tokyo, Japan) with the following primers: FR2AHC-CF (GGACTCGCCAGAGATATCAACAATATAGAC) and FR2AHC-CR (GGACTGTGAGATCGAGCGAGAC) for *FGFR2-AHCYL1* fusion, FR2BIC-CF2 (GTGTTAATGTGGGAGATCTTCACTTTAGG) and FR2BIC-CR2 (CATCCATCTTCAGTGTGACTCGATTG) for *FGFR2-BICC1* fusion, FIG-e2CF1 (ACTGGTCAAAGTGCTGACTCTGGT) and ROS-e36CR2 (CAGCAAGAGACGCAGAGTCAGTTT) for *FIG-ROS1* fusion, ACTB-S (CAAGAGATGGCCACGGCTGCT) and ACTB-A (TCCTTCTGCATCCTGTCGGCA) for *β-actin*. The PCR products were directly sequenced by Sanger sequencing using the BigDye terminator kit (Life Technologies). The expression of the *FGFR2* transcript was assayed by quantitative real-time PCR (qPCR) using the LC480 thermal cycler (Roche, Penzberg, Germany). *FGFR2* expression was normalized to β-actin expression. Primers used for qPCR are as follows: FGFR2 (Fwd-GGACCCAAAATGGGAGTTTC, Rev-ACCACTTGCCCAAAGCAA), β-actin (Fwd-CCAACCGCGAGAAGATGA, Rev-CCAGAGGCGTACAGGGATAG).

Fluorescent* In Situ *Hybridization. To identify *FGFR2* rearrangements, break-apart fluorescent *in situ* hybridization (FISH) was performed on formalin-fixed, paraffin-embedded tumors using BAC clones corresponding to the 5′ (RP11-78A18) and 3′ (RP11-7P17) sequences flanking the *FGFR2* gene and labeled by nick translation in green and red, respectively.

Immunohistochemistry. Four-micrometer-thick sections from formalin-fixed paraffin-embedded block were used for immunohistochemistry. Epitope retrieval was performed with trypsin (T7168, Sigma, St. Louis,

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MO) for 20 minutes at pH 7.7. The slides were then washed with phosphate-buffered saline (PBS) and incubated overnight with FGFR2 antibody at 4°C (1:500, ab10648, Abcam, Cambridge, UK). Immunoreactions were detected using the EnVision-FLEX system (DAKO, Glostrup, Denmark).

cDNA Cloning and Generation of Kinase-Deficient Mutants. The full-length *FGFR2-AHCYL1* and *FGFR2-BICC1* cDNAs were isolated from the corresponding tumor specimens by RT-PCR using PrimeSTAR GXL polymerase (Takara Bio) and primers FGFR2-H5F1 (ATGGTCAGCTGGGGTCGTTTCA TCTGCCTGGTCG), AHCYL-H6R1 (GTATCTGTA ATAATTAGGTTTGAATGGCCC), and BICC1-H6R1 (CCAGCGGCCACTGACACTAGCAATGTCTGA). *EZR-ROS1* cDNA was reported previously.[13] Each cDNA was subcloned into a pMXs vector (Cell Biolabs, San Diego, CA) to generate recombinant retrovirus expressing the fusion protein with a FLAG epitope tag. The kinase activity-deficient mutants were constructed by replacing tyrosine with phenylalanine at codons 568 and 569 in the *FGFR2-AHCYL1* and *FGFR2-BICC1* genes using a PrimeSTAR site-directed mutagenesis kit (Takara Bio).

Transforming Activity of FGFR2 Fusions. Mouse NIH3T3 fibroblast cells were infected with EZR-ROS1, FGFR2-AHCYL1, FGFR2-AHCYL1-KD, FGFR2-BICC1, or FGFR2-BICC1-KD-expressing retroviruses. Quantification of anchorage-independent growth was performed on day 12 in soft agar with the CytoSelect-96 kit (Cell Biolabs) in the presence or absence of FGFR inhibitors BGJ398 (#S2183, Selleck, Houston, TX) and PD173074 (#S1264, Selleck). The compound solution was added to the top layer of soft agar every 3 days.

Subcutaneous Transplantation in Immune-Compromised Mice. A total of 1×10^6 transduced NIH3T3 cells were injected subcutaneously into nude mice (BALB/c-nu/nu, CLEA Japan, Tokyo, Japan). Tumor formation was measured after 18 days. All animal procedures were performed with the approval of the Animal Ethics Committee of the National Cancer Center, Tokyo, Japan.

Immunoblot Analysis. To analyze signaling, retrovirally transduced NIH3T3 cells were serum-starved for 2 hours, after which vehicle (DMSO), BGJ398, or PD173074 was added for a further 2 hours. The culture medium was then changed to standard medium containing 10% fetal bovine serum (FBS) for 10 minutes. Whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a PVDF membrane. Western blot detection was performed with the WesternBreeze Chemiluminescent Immunodetection kit (Life Technologies) using primary antibodies against FLAG tag (#1E6, Wako Chemicals, Tokyo, Japan), phospho-FGFR1-4 (Tyr653, 654) (#AF3285, R&D Systems, Minneapolis, MN), STAT3 (#610189, BD, Becton Drive, NJ), phospho-STAT3 (Tyr705) (#9138, Cell Signaling Technology, Danvers, MA), p44/42 MAPK (#4695, Cell Signaling Technology), and phospho-p44/42 MAPK (Thr202/Tyr204) (#9106, Cell Signaling Technology), AKT1 (#2967, Cell Signaling Technology), and phospho-AKT (Ser473) (#4051, Cell Signaling Technology).

Statistical Analysis. All data analyses were performed using JMP v. 8.02 (SAS Institute, Cary, NC). Fisher's exact test was used for categorical data, and the Student *t*-test was used for continuous data. Overall survival, measured from the date of surgery, was determined using the Kaplan-Meier method, and survival difference was compared using the log-rank test. Two-sided significance level was set at $P < 0.05$.

Results

Identification of a Novel FGFR2 Fusion Gene. Whole transcriptome high-throughput sequencing of tumor specimens is one of the most effective methods for the identification of fusion oncogenes. Eight primary cholangiocarcinomas without *KRAS/BRAF* mutations or *FIG-ROS1* fusion (Supporting Table 1) were analyzed to identify novel molecular alterations by massively parallel paired-end transcriptome sequencing. Aberrant paired reads that mapped to different transcription units were identified, and 17 potential fusion transcripts were predicted by our algorithm[12] (Supporting Table 2). Sequence reads spanning the junctions of eight fusion candidate transcripts indicated in-frame gene fusion (Fig. 1A-C; Supporting Table 3) and were verified by direct sequencing of RT-PCR products spanning the breakpoints. Among these, fusion transcripts of the receptor kinase gene were detected as *FGFR2-AHCYL1*, *FGFR2-BICC1*, *AHCYL1-FGFR2*, and *BICC1-FGFR2*. However, two transcripts of *AHCYL1-FGFR2* and *BICC1-FGFR2* did not encode a functional protein of relevance to cancer, and conversely *FGFR2-AHCYL1* and *FGFR2-BICC1* were predicted to form chimeric proteins carrying the kinase domain of FGFR2 (Fig. 1D). Transcriptome sequencing showed a specific increase in the expression of the fused 3′ portion of *AHCYL1* and *BICC1* (Supporting Fig. 1A,B). Therefore, the formation of *FGFR2-AHCYL1* or *FGFR2-BICC1* might play important roles in cancer transformation.

From the tumor specimens, CC64 and CC73, we obtained cDNAs corresponding to *FGFR2-AHCYL1*

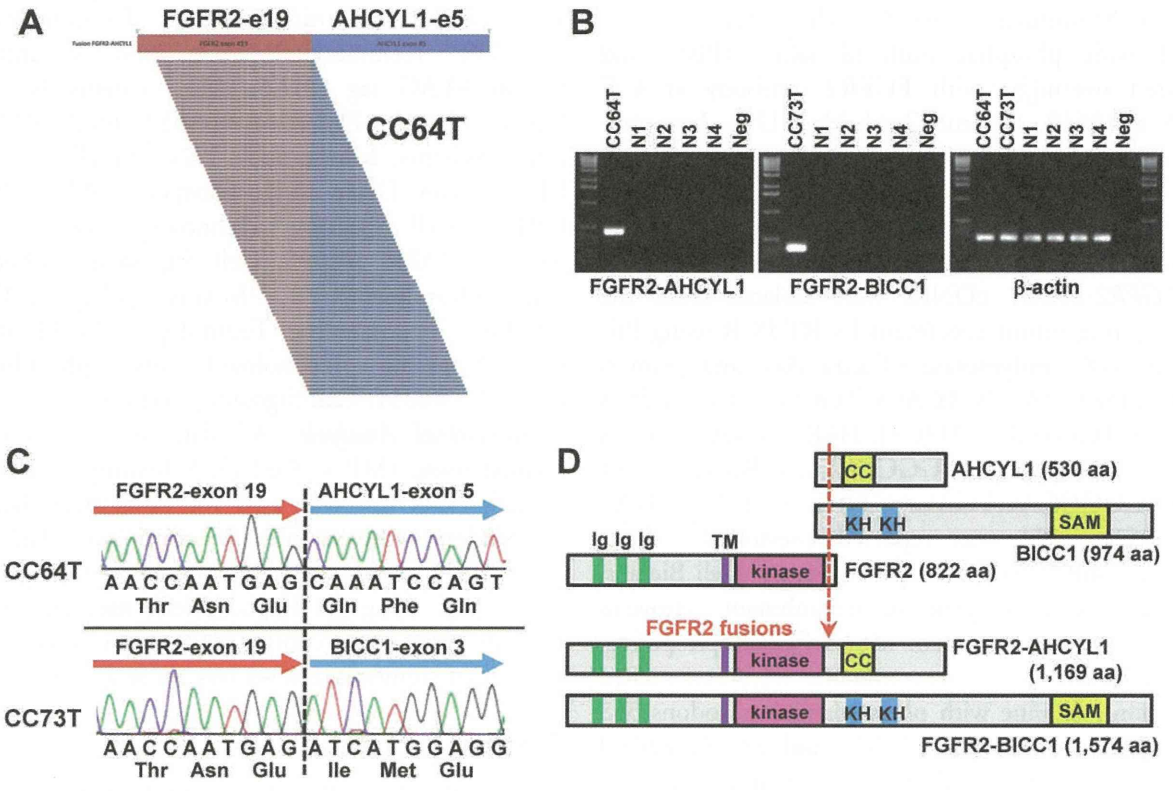

Fig. 1. *FGFR2* fusion genes in cholangiocarcinoma. (A) Junction reads representing *FGFR2-AHCYL1* fusion transcripts in CC64T samples. (B) Confirmation of tumor specific fusion transcripts by RT-PCR. Fusion transcripts were detected only in tumor tissues (CC64T and CC73T), but not in normal liver tissues (N1-N4). Neg: no template. β-Actin expression was used as a control. (C) Sanger sequencing of the RT-PCR product validated in-frame fusion transcripts. (D) Schematic representation of FGFR2-AHCYL1 and FGFR2-BICC1 fusion proteins. Ig: immunoglobulin-like domain, TM: transmembrane domain, kinase: protein tyrosine kinase domain, CC: coiled-coil domain, KH: K homology RNA binding domain, SAM: sterile alpha motif. The dotted vertical line indicates break points.

and *FGFR2-BICC1* encoding 1,169 and 1,574 amino acids, respectively. The chimeric genes consisted of the in-frame fusion of the *FGFR2* amino terminus (exons 1-19) and the *AHCYL1* carboxyl terminus (exons 5-21) or the *BICC1* carboxyl terminus (exons 3-21) (Fig. 1C,D; GenBank/DDBJ accession numbers AB821309 and AB821310). *FGFR2-AHCYL1* is a novel *FGFR2* fusion. *AHCYL1* encodes an S-adenosyl-L-homocysteine hydrolase and inositol 1,4,5-trisphosphate binding protein, and contains a coiled-coil motif in the central domain.[14] *BICC1* encodes an RNA binding protein with a sterile alpha motif (SAM) protein-interaction and dimerization module at the carboxyl terminus.[15] The FGFR2-AHCYL1 and FGFR2-BICC1 fusion proteins are likely to form homodimers through the coiled-coil motif of AHCYL1 and the SAM motif[16] of BICC1, respectively. *FGFR2*, *AHCYL1*, and *BICC1* mapped to chromosome 10q26.1, 1p13.2, and 10q21.1, respectively (Fig. 2A). *FGFR2* and *BICC1* are located on the long arm of chromosome 10 in opposite directions, suggesting that the *FGFR2-BICC1* fusion is generated by intrachromosomal inversion (Supporting Fig. 1B). Gross rearrangement of the *FGFR2* gene locus was verified by FISH with break-apart probes, which showed a split in the signals of the probes flanking the *FGFR2* breakpoint in CC64 and CC73 tumors (Fig. 2B).

Prevalence of FGFR2 Fusions. RT-PCR and Sanger sequencing analysis of 102 cholangiocarcinoma specimens (66 ICCs and 36 ECCs) from Japanese individuals, including eight who had been subjected to whole transcriptome sequencing, identified seven *FGFR2-AHCYL1*-positive and two *FGFR2-BICC1*-positive cases (Table 1; Supporting Table 4). The nine *FGFR2*-fusion-positive cases were ICC type tumors (9/66, 13.6%). *KRAS* mutations were detected in 19 cases (19/102, 17.8%) and *BRAF* mutations in one (1/102, 1%); these mutations were mutually exclusive with the *FGFR2* fusions (Fig. 3A; Supporting Table 4). Although two cases of *FIG-ROS1* fusion (2/23, 8.7%) have been reported by other researchers in cholangiocarcinoma,[10] we did not detect such fusion in this cohort. No significant differences in age, gender, tumor differentiation, clinical stage, and prognosis were detected between fusion-positive and -negative cases. (Table 2, Fig. 3B). Overall survival of ICC cases