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# Expression levels of ROS1/ALK/c-MET and therapeutic efficacy of cetuximab plus chemotherapy in advanced biliary tract cancer

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Aberrant expression of ROS1, ALK or c-MET (RAM) is implicated in carcinogenesis and cancer drug resistance. We retrospectively evaluated the effect of RAM expression on outcomes for advanced biliary tract cancer patients, who were treated with gemcitabine plus oxaliplatin (GEMOX), with or without cetuximab, in a randomized phase II trial. RAM expression levels on archived tissue sections were scored using immunohistochemistry (IHC). Of 110 tumors with IHC staining for all three markers, 18 were RAM$^{high}$ (IHC intensity 3+ for any markers). Ninety-two tumors were RAM$^{low}$ (IHC intensity <3+ for all markers). All RAM$^{high}$ tumors were intra-hepatic cholangiocarcinomas (IHCC). Of the patients with IHCC (n = 80), median overall survival (OS) of RAM$^{high}$ group was inferior to that of the RAM$^{low}$ group (5.7 *vs.* 11.7 months, $p = 0.021$). In multivariate analysis RAM$^{high}$ remained an independently adverse prognostic factor, with a hazard ratio of 2.01 ($p = 0.039$). In the RAM$^{low}$ group, GEMOX treatment with cetuximab significantly improved the disease control rate (68% *vs.* 41%, $p = 0.044$), median progression-free survival (7.3 *vs.* 4.9 months, $p = 0.026$), and marginally prolonged median OS (14.1 vs 9.6 months, $p = 0.056$), compared to GEMOX treatment alone. Future trials of anti-EGFR inhibitors for IHCC may consider RAM expression as a patient stratification factor.

Systemic chemotherapy with gemcitabine plus platinum is recognized as the standard of care for patients with advanced or metastatic biliary tract cancer (ABTC)[1]. However, treatment outcomes are largely unsatisfactory with a median overall survival (OS) ranging from 7 to 11 months[2,3]. Combination of inhibitors targeting the epidermal growth factor receptor (EGFR) pathway with chemotherapy has been extensively studied to improve the efficacy of chemotherapy[4–7]. Although trends of improved objective response rate and progression-free survival (PFS) had been observed in certain trials, none of these including "all comers" randomized trials could demonstrate the survival benefit of add-on anti-EGFR inhibitors in ABTC patients receiving gemcitabine plus platinum

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chemotherapy[4,5,7]. Identification of predictive and prognostic biomarkers is crucial to better define patients who may benefit from targeted molecular therapies such as anti-EGFR inhibitors.

Various biomarkers predictive for anti-EGFR inhibitor efficacy have been explored for different cancer types. For example, *RAS/BRAF* mutations predicted poor response to cetuximab in patients with metastatic colorectal cancers[8]. In another study, high EGFR expression predicted incremental benefit from cetuximab combine with chemotherapy in patients with advanced non-small cell lung cancer (NSCLC)[9]. However, two previous randomized studies with either pre-planned or post-hoc analyses failed to demonstrate an association between the status of *RAS/BRAF* mutations and the therapeutic efficacy of cetuximab in ABTC, or between the latter and EGFR expression[4,7]. Additional molecular alternations have been identified to account for the resistance to EGFR inhibition in NSCLC and provide opportunities for new drug development. Among them, over-expression of c-MET, which could result in activation of hepatocyte growth factor (HGF)-c-MET signaling correlates with resistance to anti-EGFR therapy in patients with NSCLC[10]. c-MET overexpression has been detected in 11.7% and 16.2% of intra- and extra-hepatic cholangiocarcinoma (IHCC and EHCC) cases, respectively, and associated with inferior post-resection recurrence-free survival than those without[11]. Chromosome rearrangements involving either anaplastic lymphoma kinase (*ALK*) or *ROS1* genes have been identified in wild-type *EGFR* NSCLCs that were refractory to anti-EGFR therapy[12,13]. *ROS1* rearrangement has been detected in 8.7% of IHCC cases and shown to be oncogenic in the BTC mouse model, whereas *ALK* rearrangement or aberrant protein expression has not been described in ABTC[14,15]. In NSCLC, *ALK* or *ROS1* rearrangement-harboring tumors exhibited compatible response to ALK inhibitors because of the high concordance of kinase domain sequences[16,17]. Whether alternations in c-MET, ROS1, and ALK signaling pathways may impact the clinical outcomes of patients undergoing anti-EGFR therapy and provide opportunities for new ABTC drug development, remains unexplored.

The present *post-hoc* study evaluates predictive and prognostic significances of aberrant ROS1, ALK or c-MET (RAM) expression in a prospective cohort of ABTC patients who received gemcitabine plus oxaliplatin (GEMOX) chemotherapy with or without cetuximab in a randomized phase II study.

## Results

**RAM protein expression and rearrangement of *ROS1* and *ALK* genes.** Of the tumor tissue sections from 122 patients who participated in the prospective randomized phase II trial, sections from 110 patients had sufficient tissue for IHC of all three markers. The staining of the RAM markers was mainly localized in the cytoplasm of tumor cells (Fig. 1A). Among them, 18 of cases (16%) were categorized as RAM^high, with 3+ IHC staining of ROS1 in 9 cases, ALK in 5, and c-MET in 6 (Supplementary Table 1). One tumor overexpressed all three markers. *ALK* rearrangement was detected in 1 of the 5 tumors presenting ALK overexpression (Fig. 1B), whereas none of the 9 tumors with ROS1 overexpression had detectable *ROS1* rearrangement.

**Correlations between RAM expression and clinicopathological factors.** As of December 31, 2014, the median follow-up was 10.3 months (range 0.9–45.4 months). The demography of the 110 ABTC patients with RAM^high or RAM^low tumors is listed in Table 1. Compared with RAM^low tumors, RAM^high tumors were more likely to be IHCC (100% *vs.* 67.4%, $p = 0.015$), without prior surgery (0% *vs.* 48.9%, $p < 0.0001$), and with concurrent mutant *KRAS* (55.6% *vs.* 31.5%, $p = 0.063$). The trend of differences in the baseline characteristics between RAM^high and RAM^low groups was similar in the 80 patients with IHCC (Supplementary Table 2).

**Comparison of treatment efficacies.** Of the 110 patients included in this study, those who received C-GEMOX had a tended to have better ORR, better long-term disease control rates (DCR$^{\geq 16wk}$, objective response according to RECIST plus stable disease [SD] $\geq$ 16 weeks), and longer survival compared to patients who received GEMOX (Supplementary Table 3)[2]. Because all RAM^high tumors were IHCCs, further analysis was performed on the IHCC population. Despite similar DCR$^{\geq 16wk}$ and PFS, IHCC patients with RAM^high tumors had a significantly inferior median OS than patients with RAM^low tumors (5.7 *vs.* 11.7 months, $p = 0.021$, Table 2). In the RAM^low IHCC subgroup, C-GEMOX treatment achieved significantly better DCR$^{\geq 16wk}$ (68% *vs.* 41%, $p = 0.044$) and median PFS (7.3 *vs.* 4.9 months, $p = 0.026$) than GEMOX alone did. A trend toward improved OS was also observed (14.1 *vs.* 9.6 months, $p = 0.056$, Table 3 and Fig. 2). Both *KRAS^wt* and *KRAS^mut* patients in the RAM^low IHCC subgroup showed similar trends of improvement in DCR$^{\geq 16wk}$ and median PFS after C-GEMOX treatment (Supplementary Table 4). This *KRAS*-independent improvement suggests that *KRAS* mutation did not interfere with the benefits derived from add-on cetuximab to GEMOX in RAM^low cases. The findings were consistent with our previous report on the entire ABTC intention-to treat population[4]. However, in the RAM^high subgroup, the DCR$^{\geq 16wk}$, PFS and OS were similar between C-GEMOX and GEMOX-treated patients (Table 3).

Multivariate analysis showed that male gender and RAM^high subgroup were independent adverse prognostic factors for OS in 80 IHCC patients (Table 4). Male gender and GEMOX alone had only a statistically non-significant tendency to prognosticate worse PFS (Supplementary Table 5).

**Recommendations for phase III trial design based on phase II trial with biomarkers.** In the RAM^low IHCC subgroup, C-GEMOX achieved better median PFS than GEMOX did, with a hazard ratio (HR) of 1.78 at one-sided $\alpha = 0.1$, and the null hypothesis was rejected, according to Freidlin *et al.*[17]. The 2-sided 80% confidence interval (CI) for the HR in the RAM^high subpopulation was 0.39 to 1.50 (Supplementary Table 6). For RAM^high cases, this range is consistent with the null hypothesis that the efficacy of C-GEMOX is no different from that of GEMOX. Therefore, future large-scale clinical trials should consider using RAM expression as a patient stratification factor.

## Discussion

The present *post-hoc* analysis is the first report to describe the prognostic significance of aberrant ROS1, ALK, or c-MET expression in ABTC patients undergoing front-line therapy. We found that patients with tumors
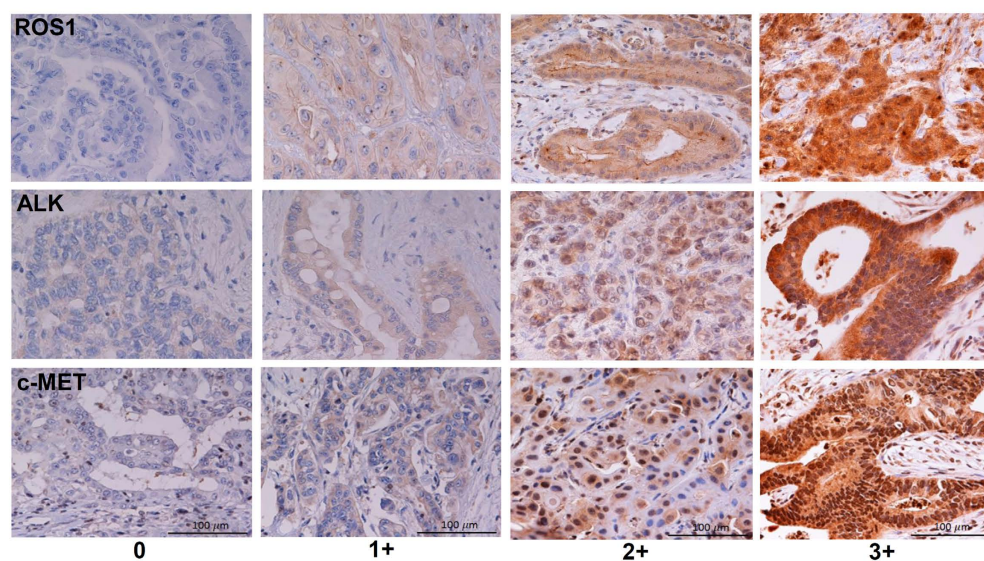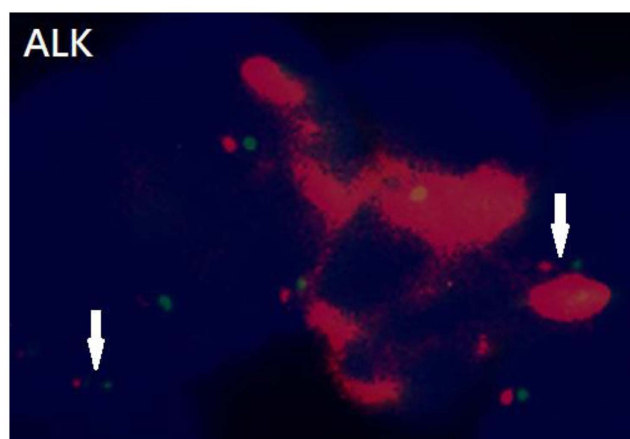
**A**



**B**



**Figure 1.** (**A**) ROS1, ALK and c-MET expression in primary intra-hepatic cholangiocarcinoma (expression score: 0, 1+, 2+ and 3+). ROS1 and ALK are mainly localized in cytoplasm, and c-MET is localized in both cytoplasm and nucleus. (**B**) Break-apart Fluorescence *in situ* hybridization for *ALK* revealed wide-apart of the red and green signals (white arrows), indicating *ALK* translocation.

over-expressing any of the ROS1, ALK, or c-MET proteins (RAM^high) had inferior OS, regardless of the assigned treatment. Patients with RAM^low tumors, notably of IHCC, were likely to benefit from the addition of cetuximab to GEMOX chemotherapy.

The findings of 18 RAM^high tumors that were all IHCC cases and without prior surgery at study entry imply that aberrant RAM expression can be site-specific and associated with aggressive tumor biology. Recent comprehensive genomic profiling studies of BTCs also suggested distinct etiologies and molecular characteristics among IHCC, EHCC and gallbladder carcinoma (GBC)[18–21]. *IDH* mutation and *FGFR* rearrangement are usually specific to IHCC, whereas *BAP1* and *IDH* mutations are more frequently detected in non-parasite related cholangiocarcinoma than in those related to parasite infection. Compared with IHCC, EHCC has a significantly higher

| | RAMhigh (n = 18) | RAMlow (n = 92) | p |
|---|---|---|---|
| Age, years | | | 1.00 |
| <60 | 9 (50%) | 47 (51%) | |
| ≥60 | 9 (50%) | 45 (49%) | |
| Sex | | | 0.61 |
| Men | 7 (39%) | 45 (49%) | |
| Women | 11 (61%) | 47 (51%) | |
| ECOG PS | | | 1.00 |
| 0 | 5 (28%) | 29 (32%) | |
| 1 | 13 (72%) | 63 (69%) | |
| Primary site | | | 0.02 |
| Intra-hepatic | 18 (100%) | 62 (67%) | |
| Extra-hepatic/GB | 0 (0%) | 30 (33%) | |
| T stage | | | 1.00 |
| ≤T$_3$ | 14 (78%) | 73 (79%) | |
| T$_4$ | 4 (22%) | 19 (21%) | |
| N stage | | | 0.39 |
| N$_0$ | 3 (17%) | 27 (29%) | |
| N$_1$ | 15 (83%) | 65 (71%) | |
| Disease status at entry | | | 0.29 |
| Locally advanced | 4 (22%) | 34 (37%) | |
| Distant metastasis | 14 (78%) | 58 (63%) | |
| Previous surgery | | | <0.001 |
| No | 18 (100%) | 45 (49%) | |
| Yes | 0 (0%) | 47 (51%) | |
| KRAS mutation | | | 0.06 |
| Negative | 8 (44%) | 63 (69%) | |
| Positive | 10 (56%) | 29 (32%) | |
| EGFR expression | | | 0.61 |
| Negative | 6 (33%) | 37 (41%) | |
| Positive | 12 (67%) | 54 (59%) | |
| HBsAg or anti-HCV Ab | | | 0.56 |
| Negative | 15 (83%) | 69 (75%) | |
| Positive | 3 (17%) | 23 (25%) | |

**Table 1. Demographic and clinical characteristic of 110 patients.** RAM, ROS1/ALK/c-MET; ECOG PS, Eastern Cooperative Oncology Group performance status; GB, gallbladder; EGFR, epidermal growth factor receptor; HBsAg, surface antigen of hepatitis B virus; HCV Ab, antibody of hepatitis C virus.

| | RAMhigh n = 18 | RAMlow n = 62 | p## |
|---|---|---|---|
| Objective response rate | 22% (6–48) | 24% (14–37) | 1.000 |
| Disease control rate# | 39% (17–64) | 53% (40–66) | 0.423 |
| Median PFS (months) | 4.5 (1.8–8.5) | 5.9 (4.2–7.1) | 0.533 |
| Median OS (months) | 5.7 (3.3–9.9) | 11.7 (8.8–14.5) | 0.021 |

**Table 2. Therapeutic efficacies stratified by RAM expression in IHCC.** RAM, ROS1/ALK/c-MET; IHCC, intrahepatic cholangiocarcinoma; PFS, progression - free survival; OS, overall survival. #Disease control rate = complete response + partial response + stable disease ≥16 weeks. ##Objective response rates and disease control rates were compared using Fisher's exact test, 2-tailed; PFS and OS were compared using log-rank tests.

frequency of *TP53* and *KRAS* mutations. These BTC subtype-specific genetic alternations indicate that studies of molecular pathogenesis and therapeutic development may be most fruitful on a subtype-specific basis. The observation of higher *KRAS* mutation rate in RAMhigh tumors indicates that the RAMhigh phenotype might be related to the 'proliferation' class of IHCC, which is associated with worse clinical outcomes than other IHCC classes[22,23].

Because of the lack of association between the status of *KRAS* mutation or EGFR positivity and therapeutic benefit of cetuximab as shown in our primary report, we strongly encourage a search for other predictive biomarkers for anti-EGFR therapy in ABTC. ROS1, ALK and c-MET, appear to be reasonable choices because the known crosstalk between EGFR signaling and all three signaling pathways, and their genetic alterations or aberrant expression may confer resistance to EGFR inhibition in NSCLC or other cancer types[24–29]. For example,

|  | C-GEMOX | GEMOX | $p^{\#\#}$ |
|---|---|---|---|
| **RAM$^{low}$** | **N = 28** | **N = 34** | |
| Disease control rate$^{\#}$ | 68% (48–84) | 41% (25–59) | 0.044 |
| Median PFS (months) | 7.3 (5.6–10.9) | 4.9 (1.8–6.7) | 0.026 |
| Median OS (months) | 14.1 (10.1–20.0) | 9.6 (6.6–13.8) | 0.056 |
| **RAM$^{high}$** | **N=10** | **N = 8** | |
| Disease control rate$^{\#}$ | 40% (12–74) | 38% (9–76) | 1.000 |
| Median PFS (months) | 4.7 (1.5–8.6) | 4.5 (1.3–13.8) | 0.610 |
| Median OS (months) | 6.3 (2.6–12.0) | 5.2 (1.4–27.4) | 0.605 |

**Table 3.  Therapeutic efficacies stratified by treatment arms in IHCC with RAM$^{low}$ and RAM$^{high}$ expression.** RAM, ROS1/ALK/c-MET; IHCC, intrahepatic cholangiocarcinoma; C-GEMOX, cetuximab plus gemcitabine and oxalin; PFS, progression-free survival; OS, overall survival. $^{\#}$Disease control rate = complete response+ partial response+ stable disease ≥16 weeks. $^{\#\#}$Disease control rates were compared using Fisher's exact test, 2-tailed; PFS and OS were compared using log-rank test.
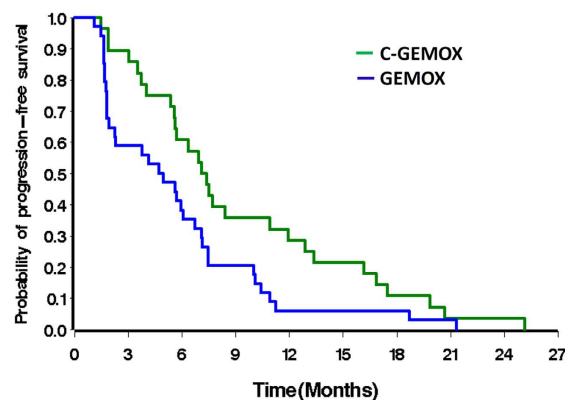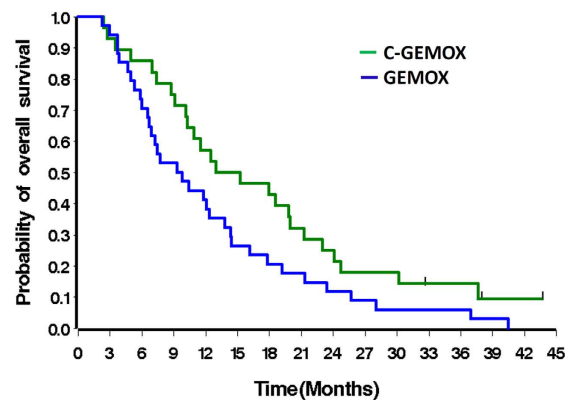


**Figure 2.** The comparison of PFS (**A**) and OS (**B**) in IHCC patients with RAM$^{low}$ -expressed tumors (n = 62), receiving treatment of C-GEMOX or GEMOX. (**C**) cetuximab; GEMOX, gemcitabine and oxaliplatin; PFS, progression-free survival; OS, overall survival; IHCC, intrahepatic cholangiocarcinoma; RAM, ROS1/ALK/c-MET.

c-MET activation involves the acquisition of resistance to anti-EGFR therapy via bypass of the EGFR pathway[26]. EGFR activation can mediate the resistance to ROS1 inhibition in NSCLC[25]. In addition, ROS1 activation mediates resistance to EGFR inhibition in glioblastoma cells[24]. ALK kinase is another druggable target in NSCLC patients who do not respond to EGFR inhibitors or have *EGFR* wild-type tumors; ALK activation may be associated with resistance to EGFR tyrosine kinase inhibitor[27,28]. Although aberrant ALK signaling has not been reported in BTC, *ROS1* rearrangement and c-MET over-expression have been detected in cholangiocarcinoma; the latter observations are associated with aggressive biological behavior[11,14,15]. For clinically available inhibitors of receptor tyrosine kinase targeting either ALK/ROS1 or ALK/ROS1 and c-MET[30,31], we include ALK expression

| Variables | Univariate | | Multivariate | |
|---|---|---|---|---|
| | Hazard ratio (95% CI) | p | Hazard ratio (95% CI) | p |
| Age ≥60 (vs. <60) | 1.48 (0.92–2.36) | 0.103 | 1.54 (0.92–2.59) | 0.102 |
| C-GEMOX (vs. GEMOX) | 0.75 (0.48–1.18) | 0.209 | – | – |
| Male (vs. female) | 1.62 (1.03–2.56) | 0.038 | 2.11 (1.27–3.50) | 0.004 |
| ECOG PS 1 (vs. 0) | 1.42 (0.87–2.34) | 0.165 | 1.27 (0.73–2.22) | 0.398 |
| Disease status, Meta (vs. LA) | 1.86 (1.12–3.09) | 0.017 | 1.35 (0.76–2.41) | 0.312 |
| Prior surgery, yes (vs. no) | 0.52 (0.32–0.85) | 0.009 | 0.81 (0.46–1.44) | 0.478 |
| KRAS MT (vs. WT) | 1.62 (1.01–2.59) | 0.045 | 1.48 (0.87–2.52) | 0.153 |
| RAM^high (vs. RAM^low) | 1.86 (1.09–3.18) | 0.023 | 2.01 (1.04–3.88) | 0.039 |

**Table 4. Univariate and multivariate Cox regression of prognostic factors for overall survival in patients with IHCC.** IHCC, intrahepatic cholangiocarcinoma; C-GEMOX, cetuximab plus gemcitabine and oxaliplatin; ECOG PS, Eastern Cooperative Oncology Group performance status; Meta: metastasis; LA, locally advanced; MT: mutated-type; WT: wild-type; LA: locally advanced RAM, ROS1/ALK/c-MET.

in our analysis. Finally, due to the low respective incidence of aberrant ROS1, ALK, or c-MET expression, a pooled analysis of all 3 molecules was performed to increase the statistical power of our analysis.

Although our previous studies demonstrated a high concordance between *ALK* rearrangement and protein over-expression in surgically resected NSCLC samples[32], FISH detected chromosomal translocation for only 1 of 5 ALK over-expression tumors in the present study (*ALK* rearrangement, Fig. 1B). However, our present finding of discord between the frequency of ROS1 over-expression and that of *ROS1* rearrangement is consistent with results of previous IHCC and NSCLC studies. Seventy-two of 194 surgically resected IHCC tumors (37%) exhibited high expression level of ROS1; none of the 102 samples successfully analyzed using FISH had detectable *ROS1* rearrangement[33]. Analyses of NSCLC tumors found that the frequency of ROS1 over-expression and *ROS1* rearrangement was 22% (9 of 399) and 1.7% (18 of 1037), respectively[12,34]. Lee *et al.* further showed that a small fraction of NSCLC tumors over-expressing ROS1 exhibited hypo-methylation in the *ROS1* promoter region; however, the mechanisms of this dysregulation remain largely un-elucidated[34]. Our discordant IHC and FISH results could be caused by the technical difficulty posed by FISH analyses of small IHCC biopsy tissue samples. Another possible explanation of the discordance could be that translational or post-translational regulation enhances the expression of ROS1/ALK proteins.

Our data indicate that RAM^low expression predicts therapeutic efficacy of add-on cetuximab in IHCC with GEMOX-based therapy. These results are applicable to this statistical model proposed by Freidlin *et al.*, which aims to clarify the values off biomarkers in designing phase III randomized trials of targeted therapies[35]. RAM expression status should be considered as a stratification factor (RAM^low *vs.* RAM^high subgroups) for future trials of anti-EGFR inhibitors in IHCC. However, the wide 80% CI of the HR estimate (Supplementary Table 6), resulting from the small sample size of patients in the RAM^high subgroup, possibly misled the conclusion according to Freidlin's algorithm. Therefore, we strongly recommend adoption of this algorithm at the interim analysis of phase III studies, through monitoring the HR of biomarker-defined subgroups. Such monitoring should be used to determine whether RAM should be used continuously as a stratification factor or shifted to the role of an enrichment factor. Based on the dismal survival outcome of RAM^high patients and the well-known clinical activity of ALK inhibitors in NSCLC with *ALK* and/or *ROS1* rearrangement, an investigator-initiated phase II trial is ongoing. This trial evaluates the efficacy and safety of ceritinib in patients with IHCC over-expressing ROS1 or ALK (clinicaltrials.gov NCT2374489).

For IHCC patients, ROS1, ALK and c-MET expression levels have prognostic significance on clinical outcomes. Although this finding may require further validation, it has led to proposal of a new stratification or enriched biomarker for future phase III trial of anti-EGFR therapy in IHCC. This example demonstrates the importance of prospective tissue sample collection from well-designed clinical trials for companion exploratory biomarker studies. Such exploratory studies can facilitate further drug development.

## Methods

**Patients.** The current study analyzed archival tumor tissues from patients with ABTC who participated in a Taiwan Cooperative Oncology Group (TCOG)-sponsored randomized phase II trial comparing first-line GEMOX alone versus GEMOX plus cetuximab (C-GEMOX)[4]. In brief, 122 patients were 1:1 randomized, stratified by tumorous *KRAS* status, ECOG performance status, and location of primary tumor, and then 1:1 randomized. The patients received GEMOX or C-GEMOX until disease progression or the occurrence of unacceptable toxicity. The primary end point of the study was objective response rate (ORR) by RECIST. Trends toward improvement in ORR (27% vs. 15%, $p = 0.12$) and median PFS (6.7 vs. 4.1 months, $p = 0.05$) favoring C-GEMOX were observed. The trends of improvement did not correlate with tumorous *KRAS* mutation status. However, C-GEMOX did not improve OS (10.6 vs. 9.8 months, $p = 0.91$) at primary analysis with a cut-off date on April 30, 2013[4]. In the present study, survival follow-up was updated to December 31, 2014. Tumor procurement was approved by the institutional review board of National Health Research Institutes (EC1040427-E). Written informed consent was obtained from all subjects and the study was conducted in accordance with International Conference Statements for Good Clinical Laboratory Practice and the declaration of Helsinki of the World Health Medical Association.

***KRAS* mutation analysis.**    Genomic DNA was extracted from tumor tissues using TaKaRa DEXPAT™ (TaKaRa Bio Inc, Japan). *KRAS* mutation status was then determined using direct sequencing of the coding sequences of exons 2 and 3, as well as the TaqMan assay for *KRAS* codon 12/13 mutations using the LightMix Kit (TIB MOLBIOL GmbH, Germany). Tumors were considered to be *KRAS* mutation if any test was positive.

**Immunohistochemical study.**    Formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections were subjected to immunohistochemistry (IHC) to determine RAM expression levels. In brief, 4-μm-thick tissue sections were de-paraffinized, followed by antigen retrieval (Cell Conditioning 1, Roche, Rotkreuz, Switzerland) for 10 minutes (for ROS1 staining) or 15 minutes (for ALK and c-MET staining) at 95 °C. The slides were then incubated with primary antibody against ROS1 (Abcam plc., Cambridge, UK, in 1:100 dilution for 1 hour), c-MET (clone C12, Biotechnology, Inc., Santa Cruz, Calif., in 1:200 dilution for 1 hour) or ALK (clone ZAL4, Invitrogen, Life Tech Corp, Carlsbad, Calif., in 1:500 dilution for 16 hours). The detection was performed by the Ventana Discovery XT staining system with ultraView Universal DAB detection kit (Ventana Medical Systems, Inc, Tucson, AZ) according to the manufacturer's protocol. Two pathologists (YYS and SFH), who were blinded to the clinical information, independently reviewed and scored the IHC staining results. The expression levels of ROS1, ALK and c-MET in tumor tissue was graded as negative (0), weak (1+), moderate (2+), or high (3+), according to the highest staining intensity identified in more than 10% of the tumor tissue. Tumors with high expression level (3+ staining intensity) of any one of the RAM markers were categorized as RAM^high tumors; those with 0 to 2+ staining intensity for all 3 markers were categorized as RAM^low tumors. EGFR protein expression was determined by immunohistochemical (IHC) staining (primary antibody: DAKO, H11 Cytomation Denmark A/S, Glostrup, Denmark), followed by staining with the ultraView Universal DAB detection kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). EGFR protein expression was considered positive if ≥1% of tumor cells were staining.

**Fluorescence *in situ* hybridization (FISH).**    Tumors over-expressing ROS1 or ALK (3+) in IHC were subjected to FISH studies as previously described[32]. The probes for detecting *ALK* and *ROS1* translocation were Vysis ALK Break Apart FISH Probe (Abbott Molecular Inc. Des Plaines, IL, USA) and ROS1 Split FISH probe (Abnova Biotec Co., Ltd, Taiwan), respectively. At least 100 intact, non-overlapping tumor nuclei were evaluated by using a Leica DMR fluorescence microscope (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Samples subjected to FISH in which more than 15% of tumor nuclei had wide apart of red and green signals for more than 2 nuclei, or an absent green signal, were considered break-apart positive. Positive break-apart samples were considered to have *ALK* or *ROS1* rearrangements.

**Decision algorithm for recommendations of phase III trial design.**    To determine whether the RAM expression status may serve as a patient enrichment or patient stratification factor in future randomized trials of EGFR inhibitors for ABTC, the median PFS of patients who received GEMOX or C-GEMOX was further analyzed using an algorithm proposed by Freidlin B *et al.* (see supplementary information for detailed methods)[35]. Briefly, the median PFS was analyzed first to test whether the GEMOX and C-GEMOX treatment groups had the same PFS in the RAM^low subpopulation. If the null hypothesis was rejected, then the 2-sided 80% CI for the HR in the RAM^high subpopulation was analyzed to determine whether RAM expression was suitable to be used as a patient-enrichment or patient-stratification biomarker in future clinical trials. If the null hypothesis was not rejected, then the 2-sided 80% CI for the HR for all patients was analyzed to determine the value of RAM expression in future clinical trials.

**Statistical Analysis.**    Correlations between the results of IHC and clinicopathological factors were assessed using Fisher's exact probability test or *t*-test. The associations between objective response rate stratified by treatment (GEMOX or C-GEMOX) and RAM expression status (RAM^high *vs* RAM^low) were evaluated using the Chi-square test or Fisher's exact test. PFS and OS were calculated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox regression was used to estimate prognostic parameters for PFS and OS. For multivariate analysis of PFS and OS, all predictors with a *p* value $< 0.2$ in univariate analysis were incorporated. Two-sided p-values less than 0.05 were considered statistically significant. All analysis was performed using the SAS 9.1 software package (SAS Institute, Inc., Cary, NC, USA).

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## Author Contributions

N.J.C., C.H. and L.T.C. wrote the manuscript; N.J.C., H.H.T., Y.Y.S. and S.F.H. performed the experiments and/or analyzed data; J.S.C., M.H.C., Y.C., T.S.Y., Y.S.S. and L.T.C. enrolled patients and collected tissue.

## Additional Information

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