Original Article

Impact of ramucirumab plus erlotinib on circulating cell-free DNA from patients with untreated metastatic non-small cell lung cancer with *EGFR*-activating mutations (RELAY phase 3 randomized study)

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Background: An exploratory, proof-of-concept, liquid biopsy addendum to examine biomarkers within cell-free DNA (cfDNA) in the RELAY phase 3, randomized, double-blind, placebo-controlled study was conducted. RELAY showed improved progression-free survival (PFS) with ramucirumab (RAM), a human immunoglobulin G1 vascular endothelial growth factor receptor 2 antagonist, plus erlotinib (ERL), a tyrosine kinase inhibitor, compared with placebo (PL) plus ERL.

Methods: Treatment-naïve patients with endothelial growth factor receptor (*EGFR*)-mutated metastatic non-small cell lung cancer were randomized (1:1) to RAM + ERL or PL + ERL. Plasma samples were collected at baseline, on treatment, and at 30-day post-study treatment discontinuation follow-up. Baseline and treatment-emergent gene alterations and *EGFR*-activating mutation allele counts were investigated by next-generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR), respectively. cfDNA concentration and fragment size were evaluated by real-time polymerase chain reaction and the BioAnalyzer. Patients with a valid baseline plasma sample were included (70 RAM + ERL, 61 PL + ERL).

Results: TP53 mutation was the most frequently co-occurring baseline gene alteration (43%). Post-study treatment discontinuation *EGFR* T790M mutation rates were 54.5% (6/11) and 41.2% (7/17) by ddPCR, and 22.2% (2/9) and 29.4% (5/17) by NGS, in the RAM + ERL and PL + ERL arms, respectively. *EGFR*-activating mutation allele count decreased at Cycle 4 in both treatment arms and was sustained at follow-up with RAM + ERL. PFS improved for patients with no detectable *EGFR*-activating mutation at Cycle 4 *vs.* those with detectable *EGFR*-activating mutation. Total cfDNA concentration increased from baseline at Cycle 4 and through to follow-up with RAM + ERL. cfDNA fragment size was similar between treatment arms at baseline [mean (standard deviation) base pairs: RAM + ERL, 173.4 (2.6); PL + ERL, 172.9 (3.2)] and was shorter at Cycle 4 with RAM + ERL *vs.* PL + ERL [169.5 (2.8) *vs.* 174.1 (3.3), respectively; P<0.0001]. Baseline *vs.* Cycle 4 paired analysis showed a decrease in cfDNA fragment size for 84% (48/57) and 23% (11/47) of patient samples in the RAM + ERL and PL + ERL arms, respectively.

Conclusions: *EGFR*-activating mutation allele count was suppressed, total cfDNA concentration increased, and short fragment-sized cfDNA increased with RAM + ERL, suggesting the additional anti-tumor effect of

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RAM may contribute to the PFS benefit observed in RELAY with RAM + ERL *vs.* PL + ERL.
Trial Registration: ClinicalTrials.gov; identifier: NCT02411448

Keywords: Next-generation sequencing (NGS); non-small cell lung cancer (NSCLC); EGFR-activating mutation alleles; circulating tumor-derived DNA; shorter cfDNA fragments

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Introduction

Activating mutations in the epidermal growth factor receptor (*EGFR*) gene are common drivers of non-small cell lung cancer (NSCLC) (1). The presence of these activating mutations has led to the development of targeted therapy for patients with *EGFR* mutation-positive NSCLC with small-molecule EGFR tyrosine kinase inhibitors (TKIs) (2,3). Despite TKIs being very effective therapy, eventually many patients will develop treatment resistance and disease progression (4,5). Therefore, new treatment that can prolong and enhance first-line EGFR TKI efficacy is desired.

Cell-free DNA (cfDNA) corresponds to DNA circulating within blood and originates from cell lysis, apoptosis, or necrosis. cfDNA consists predominantly of nucleic acids of hematopoietic origin, but in patients with cancer, cfDNA will also include circulating tumor DNA (ctDNA) derived from tumor cells (6). Tumor-derived material circulating in the blood (i.e., liquid biopsy samples) provides a less invasive alternative to tumor biopsies (7). Digital polymerase chain reaction (dPCR) and next-generation sequencing (NGS) have been used to detect and identify tumor mutational status and gene alterations from ctDNA in plasma (8,9). High concordance for the detection of T790M between tumor biopsies and cfDNA from patients with *EGFR* mutation-positive NSCLC have been observed using dPCR [83.3% (15/18)] (8) and NGS [76.2% (32/42)] (9), indicating the potential use of liquid biopsy samples for monitoring resistance mechanisms to EGFR TKI treatment. Furthermore, tracking cfDNA actively released from the tumor can be used to detect molecular residual disease, which has been shown to be associated with distant recurrence of disease; molecular residual disease may also provide lead time to disease recurrence (10).

Patterns of cfDNA size vary depending on specific conditions, such as in cancer, in the fetus, or in pregnancy (11-13). Moreover, cfDNA size distribution patterns could be useful diagnostic and prognostic markers for cancer. Research has recently demonstrated that cfDNA fragments originating from tumor cells are shorter than those from non-tumor cells (14). Generally, ctDNA fragments aggregate with cfDNA fragment lengths <150 base pairs (bp), with the median cfDNA strand length approximately 30 bp shorter in patients with cancer *vs.* those without (15). cfDNA size distribution analysis, together with cfDNA concentration measurement, can provide prognostic information in patients with advanced cancer (16-18). Differences in cfDNA size distribution are also useful in the detection of genetic abnormalities, which can then be used to guide the choice of targeted therapy. For example, in patients with hepatocellular carcinoma, single bp resolution sequencing studies indicate that shorter plasma cfDNA fragments were more likely to include copy number alterations associated with the tumor than longer

Highlight box

Key findings

- In this study of patients with *EGFR*-mutated metastatic non-small cell lung cancer, *EGFR*-activating mutation allele count was suppressed, total circulating cell-free DNA (cfDNA) concentration increased, and short fragment-sized cfDNA increased with combined ramucirumab and erlotinib treatment.

What is known and what is new?

- The RELAY study demonstrated that ramucirumab plus erlotinib improved progression-free survival compared with placebo plus erlotinib in patients with treatment-naïve *EGFR*-mutated metastatic non-small cell lung cancer.
- This exploratory liquid biopsy addendum of RELAY investigated whether ramucirumab plus erlotinib affects changes in cfDNA levels and fragment size.

What is the implication, and what should change now?

- These results suggest that the additional anti-tumor effect of ramucirumab on tumor and non-tumor cells may contribute to the progression-free survival benefit observed in the RELAY study.

cfDNA fragments (19); however, for some patients with cancer with a low mutant allele frequency, longer cfDNA fragments have been observed (20). Therefore, cfDNA size distribution could be used as a biomarker for prognosis and for the elucidation of mechanisms of action in clinical studies.

We conducted an exploratory liquid biopsy study as part of the RELAY global, phase 3, randomized, double-blind, placebo-controlled study, which investigated the efficacy and safety of ramucirumab (RAM), a human immunoglobulin G1 vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) antagonist, plus erlotinib (ERL), an EGFR TKI, in treatment-naïve patients with *EGFR*-mutated metastatic NSCLC (21). A significant improvement in progression-free survival (PFS) with RAM + ERL compared with placebo (PL) plus ERL was observed {median PFS: 19.4 *vs.* 12.4 months; hazard ratio (HR): 0.59 [95% confidence interval (CI): 0.46–0.76; P<0.0001]}.

The objective of this RELAY exploratory, proof-of-concept, liquid biopsy addendum, specific to patients enrolled in Japan only, was to investigate (using liquid biopsy samples) whether the combination of RAM + ERL affects the occurrence of the *EGFR* T790M mutation and/or other mutations related to acquired EGFR TKI resistance, the association between biomarkers and treatment outcome, and the changes in cfDNA levels and fragment size. We hypothesized that cfDNA size distribution could help elucidate the mode of action of RAM in combination with an EGFR TKI in this clinical study. We present this article in accordance with the CONSORT reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-736/rc).

Methods

Study design and patients

The study design and patient population for the RELAY study have been previously described (ClinicalTrials.gov identifier: NCT02411448) (21). Briefly, RELAY was a global, phase 3, randomized, double-blind, placebo-controlled study of patients with untreated *EGFR*-mutated metastatic NSCLC. Two exploratory biomarker studies were conducted in RELAY; the first was conducted in the global intent-to-treat (ITT) population including Japanese patients, and the second was an optional exploratory, proof-of-concept, liquid biopsy addendum specific to RELAY patients enrolled in Japan only. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), the Council for International Organizations of Medical Sciences International Ethical Guidelines, Good Clinical Practice guidelines, and local guidelines. The protocol and addendum were approved by the ethics review boards at each site (details are provided in Table S1) and all patients provided written informed consent; patients who opted to participate in the exploratory liquid biopsy addendum provided additional consent.

Study population

Eligibility criteria for the RELAY phase 3 study have been previously published (21). Briefly, patients were ≥18 years of age (≥20 years in Japan and Taiwan) and had stage IV NSCLC, documented evidence of the *EGFR* exon 19 deletion (ex19del) mutation or the exon 21 L858R point mutation (ex21.L858R), ≥1 measurable lesion attributed to NSCLC [defined by Response Evaluation Criteria in Solid Tumours version 1.1 (RECIST v1.1)], Eastern Cooperative Oncology Group performance status of 0 or 1, and adequate organ function. Patients were excluded if they had received previous anti-cancer treatment for stage IIIb/IV NSCLC (except previous radiation therapy), had central nervous system metastases, or had a documented T790M *EGFR* mutation.

Treatment

Eligible patients were randomized (1:1) to RAM + ERL (RAM: 10 mg/kg intravenously every 2 weeks; ERL: 150 mg orally once daily) or PL + ERL (PL: intravenously every 2 weeks; ERL: 150 mg orally once daily) and were assigned using an interactive web response system. A treatment cycle was defined as 2 weeks. Patients received study treatment until disease progression, unacceptable toxicity, non-compliance, or investigator or patient decision.

Outcome measures

The primary endpoint of the RELAY phase 3 randomized portion was PFS according to RECIST v1.1. PFS was defined as the time from randomization to disease progression or death from any cause. Secondary endpoints included objective response rate (ORR), disease control rate (DCR), and duration of response (DoR), as previously described (21). Exploratory endpoints of the RELAY exploratory liquid biopsy addendum, specific to patients

enrolled in Japan only, included co-occurring gene alterations at baseline detected by NGS and their potential impact on treatment outcomes (PFS, ORR, DCR, DoR) and EGFR TKI resistance mechanisms, T790M mutation rates post-study treatment discontinuation detected by droplet dPCR (ddPCR), and changes in cfDNA concentration and cfDNA fragment size throughout treatment.

Liquid biopsy sample collection and analysis

Plasma samples from patients who opted to participate in the exploratory liquid biopsy addendum study were collected for cfDNA assessment at baseline, during treatment (Cycle 4, Cycle 13, and every 6 cycles thereafter), and at 30-day post-study treatment discontinuation follow-up. Gene alterations were assessed at baseline, Cycle 4, and 30-day follow-up by NGS using the Ion AmpliSeq Colon and Lung Cancer Panel v2 (Thermo Fisher Scientific, Waltham, MA, USA). Genes included within the panels were the following: *KRAS*, *EGFR*, *BRAF*, *PIK3CA*, *AKT1*, *ERBB2*, *PTEN*, *NRAS*, *STK11*, *MAP2K1*, *ALK*, *DDR2*, *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBXW7*, *FGFR3*, *NOTCH1*, *ERBB4*, *FGFR1*, and *FGFR2*. For library preparation, cfDNA (maximum of 3,000 copies) was subjected to multiplex PCR amplification, and purified libraries were pooled and sequenced with an Ion S5 XL NGS platform and 550™ Chip Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reads were aligned with the hg19 human reference genome; germline mutations were excluded with the use of the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB) (22). Potential mutations were called using Variant Call Format version 5.12, as previously described (23). *EGFR*-activating mutation allele count was evaluated at all time points by ddPCR; 6 µL of cfDNA (maximum of 3,000 copies) template was added per 20 µL of ddPCR. Assays were performed in duplicate. All ddPCR assays included negative template controls and positive template controls in triplicate. Plasma cfDNA concentrations were quantified using the TaqMan RNase P Detection Reagents with the StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA); 1 µL of cfDNA template was subjected to real-time PCR, and cfDNA copy number was determined in reference to a standard curve. cfDNA fragment size was analyzed using the High Sensitivity DNA Chips and the 2100 Bioanalyzer Expert Software package on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Size was determined from an external standard ladder (DNA sizing ladder), ranging from 50 up to 7,000 bp.

Statistical analysis

The analyses in this report were exploratory. The data cut-off dates were January 23, 2019 (efficacy results) and September 30, 2021 (exploratory liquid biopsy addendum results). The exploratory liquid biopsy addendum translational research (TR) population comprised patients with available baseline NGS or ddPCR results. The TR subpopulations consisted of patients with a valid baseline sample in which ≥1 gene alteration was detected by NGS (TR-NGS) or ddPCR (TR-ddPCR). Baseline gene alterations are reported for patients with any detectable gene alteration at baseline. Treatment-emergent gene alterations are reported for patients with any detectable alteration at baseline and at 30-day post-study treatment discontinuation. PFS and DoR were analyzed using the Kaplan-Meier method and compared using the unstratified log-rank test. An analysis of covariance was conducted separately for cfDNA fragment size and log-transformed cfDNA concentration. Each analysis was done by treatment arm and visit for baseline, Cycle 4, and follow-up. Post-hoc pairwise analyses with Bonferroni adjustments applied were used to compare fragment size between treatment arms within the same treatment visit and to compare across treatment visits within the treatment arm. The relationship between change in cfDNA concentration and change in cfDNA fragment size from baseline to Cycle 4 (i.e., during the early stage of study treatment) was evaluated by Pearson's correlation coefficient for the linear relationship and Spearman's rank correlation coefficient for the monotonic relationship.

Results

Demographic and baseline clinical characteristics

The RELAY Japanese ITT population comprised 211 patients (RAM + ERL: 106 patients; PL + ERL: 105 patients) enrolled at 41 sites (24). Of these 211 patients, 136 participated in the optional exploratory liquid biopsy addendum, which required frequent liquid biopsy sampling (Figure S1). The TR population comprised 131 patients with valid baseline (NGS and ddPCR) assay results. The TR-NGS (N=84) and TR-ddPCR (N=74) populations consisted of patients with ≥1 gene alteration detected at

Table 1 Baseline patient and disease characteristics for the TR-NGS and TR-ddPCR populations

Variable	TR-NGS[†] (N=84)	TR-ddPCR[†] (N=74)	Japan ITT[‡] (N=211)
Sex, n (%)			
Female	58 (69.0)	48 (64.9)	140 (66.4)
Age, n (%)			
<65 years	32 (38.1)	26 (35.1)	79 (37.4)
≥65 years	52 (61.9)	48 (64.9)	132 (62.6)
Smoking history[§], n (%)			
Ever	25 (29.8)	23 (31.1)	62 (29.4)
Never	50 (59.5)	45 (60.8)	127 (60.2)
ECOG PS 0, n (%)	43 (51.2)	36 (48.6)	119 (56.4)
EGFR mutation[¶], n (%)			
Exon 19 deletion	44 (52.4)	32 (43.2)	100 (47.4)
Exon 21 L858R mutation	40 (47.6)	42 (56.8)	110 (52.1)

[†], population consists of patients from whom a valid baseline sample with ≥1 alteration detected has been obtained; [‡], previously reported in Nishio *et al.*, 2021 (24); [§], smoking history was unknown or missing for 22/211 (10%) patients in the Japan ITT population, 9/84 (11%) patients in the TR-NGS population, and 6/74 (8%) patients in the TR-ddPCR population; [¶], information on *EGFR* mutation was missing for 1 patient in the RAM + ERL group of the Japan ITT population. TR, translational research; NGS, next-generation sequencing; ddPCR, droplet digital polymerase chain reaction; ITT, intent-to-treat; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; RAM, ramucirumab; ERL, erlotinib.

Table 2 Summary of efficacy endpoints in the TR population

Response	TR population		
	RAM + ERL (N=70)	PL + ERL (N=61)	Overall (N=131)
CR	1 (1.4)	0	1 (0.8)
PR	53 (75.7)	44 (72.1)	97 (74.1)
SD	12 (17.1)	15 (24.6)	27 (20.6)
Progressive disease	1 (1.4)	1 (1.6)	2 (1.5)
Not assessed	3 (4.3)	1 (1.6)	4 (3.1)
ORR (CR + PR)	54 (77.1)	44 (72.1)	98 (74.8)
DCR (CR + PR + SD)	66 (94.3)	59 (96.7)	125 (95.4)
Duration of response[†]			
Events	29 (53.7)	33 (75.0)	
Median (95% CI) (months)	19.0 (15.0–NA)	11.1 (9.0–16.5)	
HR (95% CI)	0.51 (0.31–0.84)		

Data are n (%) except where indicated. [†], in patients who responded (RAM + ERL: n=54; PL + ERL: n=44). TR, translational research; RAM, ramucirumab; ERL, erlotinib; PL, placebo; CR, complete response; PR, partial response; SD, stable disease; ORR, objective response rate; DCR, disease control rate; CI, confidence interval; NA, not available; HR, hazard ratio.

baseline (confirming the presence of ctDNA) by either NGS or ddPCR, respectively (Figure S1). Patients could belong to both the TR-NGS and TR-ddPCR populations; 61 patients were in both populations (*EGFR* ex19del: 30 patients; *EGFR* ex21.L858R: 31 patients). Patient and disease characteristics for the TR-NGS and TR-ddPCR populations were similar to the RELAY Japanese ITT population (*Table 1*). In both TR populations, most (>60%) patients were female, most (>61%) were ≥65 years of age, and >59% had never smoked. *EGFR* ex21.L858R mutations were identified in 40 patients in the TR-NGS group and in 42 patients in the TR-ddPCR group.

Efficacy

In the overall TR population, median PFS was 20.8 *vs.* 12.5 months [HR: 0.61 (95% CI: 0.38–0.97)] in the RAM + ERL *vs.* PL + ERL arms, respectively, similar to that observed in the RELAY ITT (21) and Japanese ITT (24) populations (Figure S2). Furthermore, median DoR was longer with RAM + ERL *vs.* PL + ERL [19.0 *vs.* 11.1 months; HR: 0.51 (95% CI: 0.31–0.84)] (*Table 2*) and was similar to that observed in the Japanese ITT population (24).

Co-occurring gene alterations

Co-occurring baseline gene alterations detected by NGS in ≥5 patients in the TR-NGS population were *TP53* (36/84 patients, 42.9%), *PTEN* (6/84 patients, 7.1%), and *KRAS* (5/84 patients, 6.0%) (*Table 3*). Comparison of PFS by baseline *TP53* status and treatment arm did not establish a prognostic or predictive relationship (data not shown).

Treatment-emergent gene alterations detected by NGS at post-study treatment discontinuation included *EGFR*, *FGFR3*, *KRAS*, and *TP53* (*Table 4*). Treatment-emergent *EGFR* mutations included T790M (by NGS and ddPCR) and H870R (assessed by NGS only) (*Table 4*). T790M

Table 3 Co-occurring baseline gene alterations detected in liquid biopsy samples by NGS using the Ion AmpliSeq Colon and Lung Cancer Panel in the TR-NGS population

Gene	RAM + ERL (N=41), n (%)	PL + ERL (N=43), n (%)	Overall (N=84), n (%)
TP53	18 (43.9)	18 (41.9)	36 (42.9)
PTEN	1 (2.4)	5 (11.6)	6 (7.1)
KRAS	3 (7.3)	2 (4.7)	5 (6.0)
Other *EGFR*[†]	2 (4.9)	2 (4.7)	4 (4.8)
T790M	1 (2.4)	0	1 (1.2)
CTNNB1	0	3 (7.0)	3 (3.6)
MET	2 (4.9)	0	2 (2.4)
BRAF	1 (2.4)	0	1 (1.2)
FBXW7	0	1 (2.3)	1 (1.2)
FGFR3	1 (2.4)	0	1 (1.2)
PIK3CA	0	1 (2.3)	1 (1.2)
SMAD4	0	1 (2.3)	1 (1.2)

[†], *EGFR* mutation excluding *EGFR*-activating mutations exon 19 deletion and exon 21 L858R mutation. NGS, next-generation sequencing; TR, translational research; RAM, ramucirumab; ERL, erlotinib; PL, placebo; EGFR, epidermal growth factor receptor.

Table 4 Treatment-emergent gene alterations detected in liquid biopsy samples collected at post-study treatment discontinuation visit

Gene alterations	RAM + ERL, n (%)	PL + ERL, n (%)	Overall, n (%)
Mutations detected by NGS[†]			
EGFR[‡]	2 (22.2)	5 (29.4)	7 (26.9)
H870R	1 (11.1)	0	1 (3.8)
T790M	2 (22.2)	5 (29.4)	7 (26.9)
FGFR3	1 (11.1)	0	1 (3.8)
KRAS	2 (22.2)	0	2 (7.7)
TP53	3 (33.3)	1 (5.9)	4 (15.4)
None	4 (44.4)	11 (64.7)	15 (57.7)
Mutations detected by ddPCR[§]			
T790M	6 (54.5)	7 (41.2)	13 (46.4)

[†], RAM + ERL, N=9; PL + ERL, N=17; Overall, N=26; [‡], one patient had 2 treatment-emergent *EGFR* mutations (T790M and H870R); 3 patients did not have *EGFR*-activating mutations detected in ctDNA at baseline but did have these mutations detected at 30-day post-study treatment discontinuation, consistent with their local baseline testing (2 exon 19 deletion, 1 L858R), and were not included in this treatment-emergent summary; [§], RAM + ERL, N=11; PL + ERL, N=17; Overall, N=28. RAM, ramucirumab; ERL, erlotinib; PL, placebo; NGS, next-generation sequencing; ddPCR, droplet digital polymerase chain reaction; EGFR, epidermal growth factor receptor; ctDNA, circulating tumor DNA.

mutation rates by ddPCR were 54.5% (6/11 patients) in the RAM + ERL arm and 41.2% (7/17 patients) in the PL + ERL arm; T790M mutation rates by NGS were 22.2% (2/9 patients) in the RAM + ERL arm and 29.4% (5/17 patients) in the PL + ERL arm.

EGFR-activating mutant alleles and treatment outcome

When assessed according to dichotomized baseline *EGFR*-activating mutation allele count in this data set (low or high allele count was below or above the baseline median mutation allele count of 102, respectively), no difference in PFS was observed in either treatment arm (*Figure 1A*). The number of *EGFR*-activating mutation alleles decreased from baseline at Cycle 4 and was sustained throughout treatment in both treatment arms; at follow-up, allele count increased in the PL + ERL arm but not in the RAM + ERL arm (Figure S3). At baseline, an *EGFR*-activating mutation was detectable in 94.6% (35/37) of patients in the RAM + ERL arm and 94.6% (35/37) of patients in the PL + ERL arm. At Cycle 4, no *EGFR*-activating mutation was detectable in 78% (21/27) of patients in the RAM + ERL arm and 67% (20/30) of patients in the PL + ERL arm. PFS was improved for patients with no detectable *EGFR*-activating mutation at Cycle 4 (median PFS: RAM + ERL: not reached *vs.* PL + ERL: 12.52 months; HR: 0.28, 95% CI: 0.09–0.73) *vs.* those with detectable *EGFR*-activating mutation (median PFS: RAM + ERL, 12.65 months *vs.* PL + ERL, 9.64 months; HR: 0.66, 95% CI: 0.14–2.28) (*Figure 1B*).

Total cfDNA concentration and fragment size

Total cfDNA concentration increased in the RAM+ERL arm from Cycle 4 and was sustained throughout treatment [mean (standard deviation): baseline, 92.1 (52.58) *vs.* Cycle 4, 239.4 (144.96) copies/μL, P for log-transformed data <0.0001; baseline *vs.* follow-up: 314.6 (512.38) copies/μL, P for log-transformed data <0.0001] (*Figure 2*). cfDNA fragment size was similar at baseline in the 2 treatment arms [mean (standard deviation): RAM + ERL, 173.4 (2.6) *vs.* PL

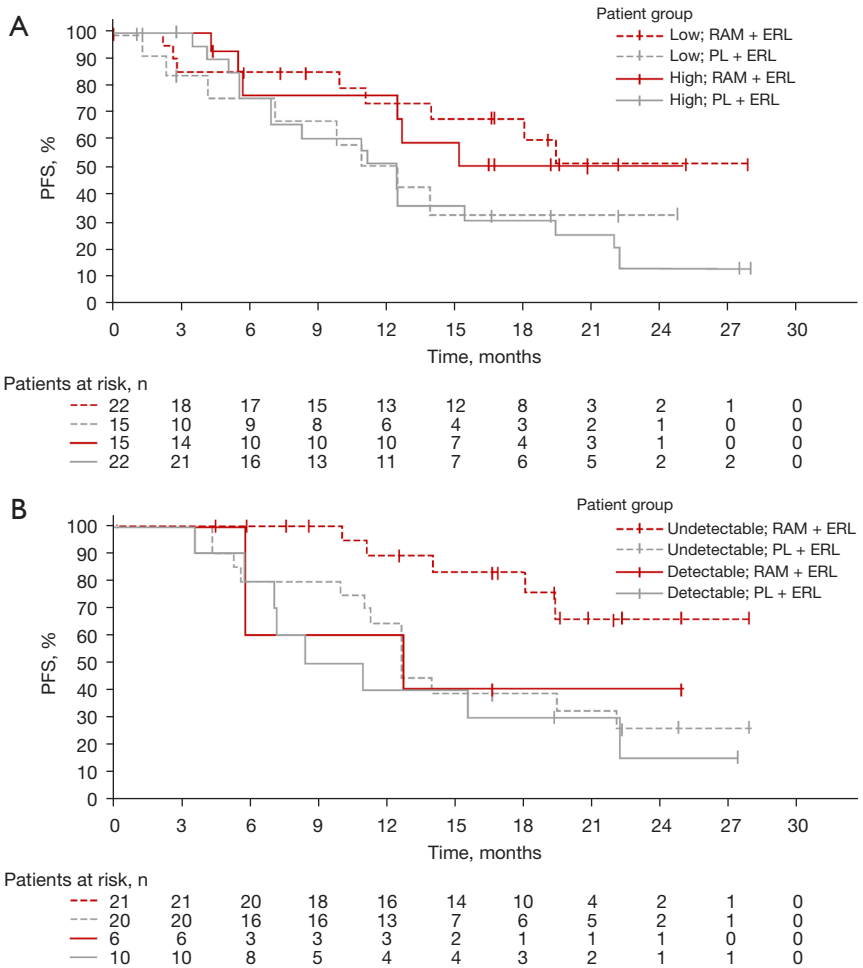


Figure 1 Kaplan-Meier plots of investigator-assessed PFS in the exploratory liquid biopsy addendum by (A) dichotomized baseline circulating *EGFR*-activating mutation (ex19del or ex21.L858R) allele count (low *vs.* high; subgroups based on the count of *EGFR*-activating mutation alleles at baseline above or below the median count of 102) and (B) Cycle 4 circulating *EGFR*-activating mutation (ex19del or ex21.L858R) alleles (undetectable *vs.* detectable). EGFR, epidermal growth factor receptor; ERL, erlotinib; ex19del, exon 19 deletion; ex21.L858R, exon 21 L858R point mutation; PFS, progression-free survival; PL, placebo; RAM, ramucirumab.

+ ERL, 172.9 (3.2) bp; P=0.35] but was shorter in the RAM + ERL arm compared with the PL + ERL arm at Cycle 4, respectively [mean (standard deviation): 169.5 (2.8) *vs.* 174.1 (3.3) bp; P<0.0001] (*Figure 3A*). No change in pattern for subgroups by *EGFR*-activating mutation (detected/not detected) at baseline was observed (data not shown). cfDNA fragment size decreased (baseline *vs.* Cycle 4) in 84% (RAM + ERL; 48/57) and 23% (PL + ERL; 11/47) of paired patient samples (*Figure 3B*). A trend for shorter cfDNA fragment size in the RAM + ERL arm compared with the PL + ERL arm was observed throughout treatment and at follow-up (*Figure 4*). A negative correlation between change in cfDNA concentration *vs.* change in cfDNA fragment size from baseline to Cycle 4 was identified in the overall TR population (RAM + ERL and PL + ERL treatment arms combined) (*Figure 5*).

Discussion

This exploratory liquid biopsy addendum of the RELAY phase 3 study of RAM + ERL *vs.* PL + ERL examined gene alterations, total cfDNA concentration, and cfDNA fragment size in patient-derived liquid biopsy samples throughout and after treatment. Improvement in PFS was observed for patients with no detectable *EGFR*-activating mutation at Cycle 4 in liquid biopsy samples compared

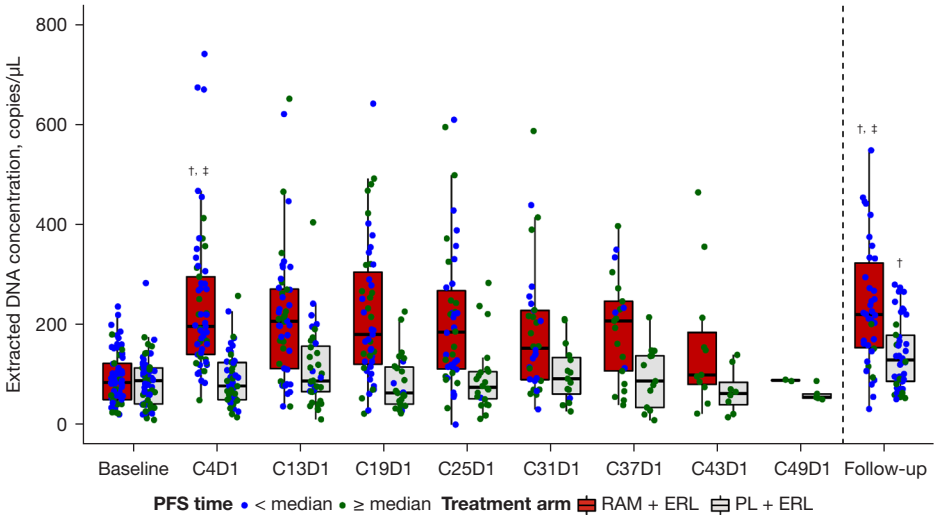
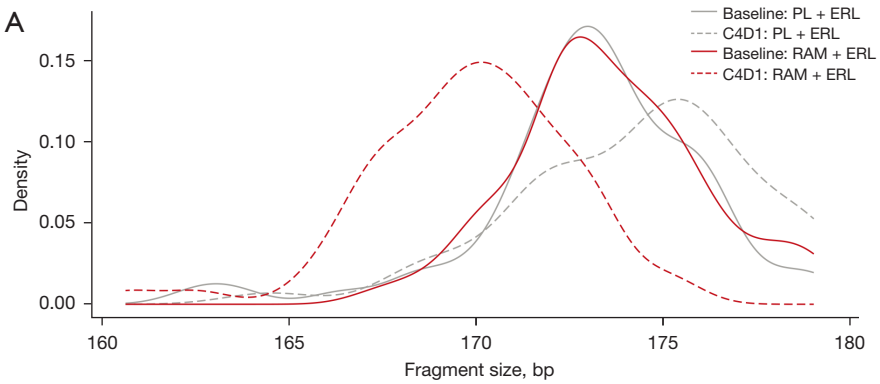


Figure 2 Total cfDNA concentration by treatment arm (TR population, patients with a valid baseline sample). Population eligibility required the presence of a valid baseline sample only. Patients were dichotomized by median PFS time within treatment arm separately. Dots represent individual patient data. One patient had an extracted DNA concentration of 3,302.1 copies/µL at follow-up; this data point was removed from the plot. †, P for log-transformed data <0.0001 *vs.* baseline within treatment arm; ‡, P for log-transformed data <0.0001 RAM + ERL *vs.* PL + ERL at time point. C, Cycle; D, Day; PFS, progression-free survival; RAM, ramucirumab; ERL, erlotinib; PL, placebo; cfDNA, cell-free DNA; TR, translational research.

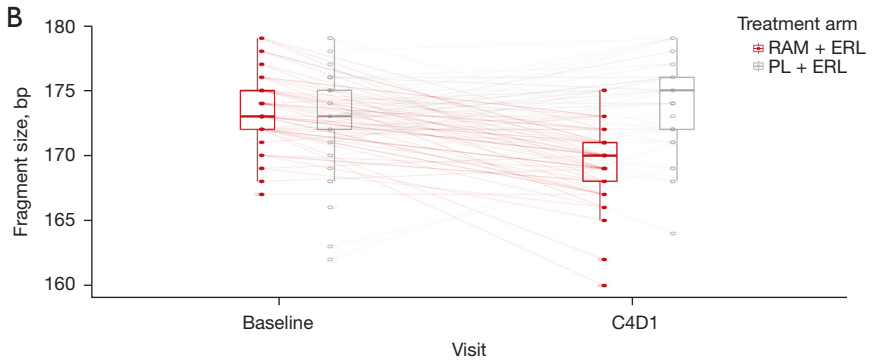
with those with a detectable *EGFR*-activating mutation. Furthermore, throughout treatment, increased levels of total cfDNA were detected in the RAM + ERL patient samples but were not apparent in the PL + ERL patient samples, suggesting an enhanced anti-tumor effect with the addition of RAM to ERL. Fragment size of the total cfDNA content in the RAM + ERL samples was shorter than that in the PL + ERL samples, suggesting that the increased total cfDNA levels in the RAM + ERL arm are likely due to increased tumor cell apoptosis. These results provide insight into possible mechanisms of resistance and/or efficacy of RAM in addition to ERL in the treatment of NSCLC.

EGFR mutation-positive tumors often contain co-occurring gene alterations, the identification of which will vary depending on the detection method used (25). Common co-occurring gene alterations include *TP53*, *PI3KCA*, *RB1*, or *CTNNB1* and will vary in frequency in early-stage *vs.* advanced-stage tumors (26). *TP53*, *PI3KCA*, and *RB1* co-occurring alterations have a prognostic impact on worse clinical outcomes in *EGFR* mutation-positive NSCLC treated with EGFR TKI therapy (26-29). In this study of Japanese patients with *EGFR* mutation-positive NSCLC, common baseline gene alterations co-occurring with an *EGFR*-activating mutation in ≥5 patients included *TP53*, *PTEN*, and *KRAS*. Treatment-emergent gene alterations included *EGFR* T790M and *TP53* in both treatment arms, and *EGFR* H870R, *FGFR3*, and *KRAS* in the RAM + ERL arm. One patient harbored the previously described *EGFR* H870R mutation that in combination with ex21.L858R may lead to resistance to the EGFR TKI gefitinib (30,31).

Of patients receiving first- or second-generation EGFR TKI therapy for the treatment of *EGFR* mutation-positive NSCLC, 50–60% will acquire the *EGFR* T790M resistance mutation (32), after which the only effective EGFR TKI therapy available is a third-generation EGFR TKI (2,3). High sensitivity and quantitative concordance of amplicon-based plasma NGS compared with ddPCR in detecting resistance mechanisms, such as T790M, have been demonstrated (33). In the RELAY global ITT and Japanese ITT populations, post-progression T790M rates detected by Guardant 360 NGS were similar for RAM + ERL *vs.* PL + ERL (21,24). In this exploratory liquid biopsy study, T790M rates detected by ddPCR and NGS at post-study treatment discontinuation differed, which may have been due to the different sensitivity of the detection methods. Regardless of the testing method used, the observed cumulative frequency of the T790M mutation detected in liquid biopsy samples was not affected by the addition of



cfDNA fragment size	Baseline		C4D1	
	RAM + ERL	PL + ERL	RAM + ERL	PL + ERL
N	70	61	57	47
Mean (SD), bp	173.4 (2.6)	172.9 (3.2)	169.5 (2.8)	174.1 (3.3)
Median, bp	173	173	170	175
Q1–Q3, bp	172–175	172–175	168–171	172–176
Min-max, bp	167–179	162–179	160–175	164–179



Change in fragment size	RAM + ERL, N (%)	PL + ERL, N (%)
Increased	6 (11)	28 (60)
Stable	3 (5)	8 (17)
Decreased	48 (84)	11 (23)

Figure 3 Distribution of cfDNA fragment size (TR population, patients with a valid baseline sample). (A) Distribution of total cfDNA fragment size at baseline and at Cycle 4. (B) Baseline *vs.* Cycle 4, paired samples. PL, placebo; ERL, erlotinib; C, Cycle; D, Day; RAM, ramucirumab; cfDNA, cell-free DNA; SD, standard deviation; bp, base pairs; Q, quartile; max, maximum; min, minimum; TR, translational research.

RAM to ERL. These results suggest that first-line RAM + ERL could provide the opportunity for second-line molecular targeted therapy by third-generation EGFR TKI treatment that is active against the T790M mutation.

EGFR-activating mutation allele count/allele frequency in liquid biopsy samples could be a potential biomarker for response to treatment. Buder *et al.* (34) showed that patients with advanced *EGFR* T790M-mutated NSCLC who responded to second-line osimertinib had a significantly lower *EGFR*-activating mutation allele frequency at baseline than patients who did not respond, and that a higher allele frequency in plasma ctDNA was associated with shorter

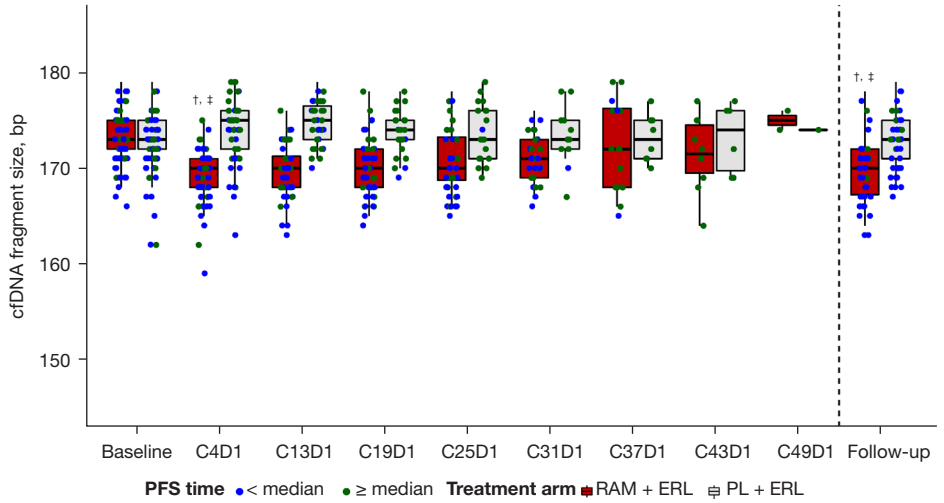


Figure 4 cfDNA fragment size over time (TR population, patients with a valid baseline sample). Patients are dichotomized by median PFS time within treatment arm. Dots represent individual patient data. †, P<0.0001 *vs.* baseline within treatment arm; ‡, P<0.0001, RAM + ERL *vs.* PL + ERL at time point. cfDNA, cell-free DNA; bp, base pairs; PFS, progression-free survival; C, Cycle; D, Day; RAM, ramucirumab; ERL, erlotinib; PL, placebo; TR, translational research.

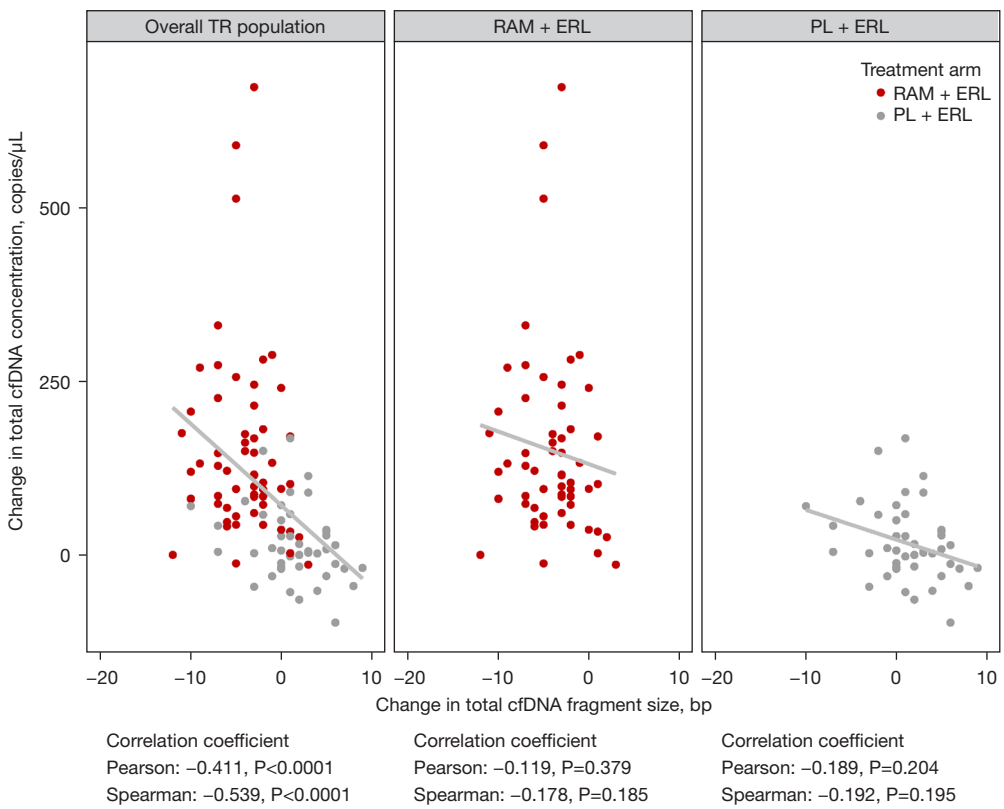


Figure 5 Relationship between change in total cfDNA concentration and change in total cfDNA fragment size from baseline to Cycle 4 (TR population, patients with a valid baseline sample). RAM + ERL, N=57; PL + ERL, N=47. cfDNA, cell-free DNA; TR, translational research; RAM, ramucirumab; ERL, erlotinib; PL, placebo; bp, base pairs.

PFS. In our study, no difference in PFS was observed between high and low baseline count of *EGFR*-activating mutation alleles with RAM + ERL or PL + ERL treatment. However, PFS was improved for patients with no detectable *EGFR*-activating mutation alleles at Cycle 4 compared with those who did have detectable *EGFR*-activating mutation alleles. These results suggest that monitoring of circulating *EGFR*-activating mutation alleles at Cycle 4 could provide insights regarding likely clinical benefit. Although the count of *EGFR*-activating mutation alleles at baseline was slightly different between treatment arms, it was suppressed to a similar level throughout treatment and was only sustained at post-study treatment discontinuation follow-up with RAM + ERL treatment, suggesting that RAM enhances the sustained anti-tumor effect of ERL on *EGFR*-mutated cells. From the viewpoint of molecular residual disease detection, monitoring the *EGFR*-activating mutation fraction by liquid biopsy may predict disease progression; however, further investigation is required for the clinical application of such monitoring.

The total cfDNA content is the sum of tumor-derived ctDNA, such as ctDNA from ex19del- or ex21.L858R-mutated tumor cells and other tumor cells, cfDNA from normal cells in the tumor microenvironment (e.g., stroma and pericytes), and cfDNA from primary hematopoietic origin, with ctDNA fragment lengths frequently being shorter than normal cfDNA fragments (35). Moreover, ctDNA and cfDNA are considered to be real-time snapshots of the tumor microenvironment due to their short half-lives. However, we need to interpret cfDNA profiling carefully because there are 2 different mechanisms by which cfDNA is released into the bloodstream; one is active release of cfDNA from the tumor and the other is passive release from dying cells by treatment (36,37). In our analysis, despite the number of *EGFR*-activating mutation alleles being suppressed at Cycle 4 and throughout treatment in both treatment arms, increased levels of total cfDNA were detected throughout treatment in the RAM+ERL arm but not in the PL + ERL arm. Furthermore, fragment size of the total cfDNA content in the RAM + ERL arm was shorter than at baseline and then in the PL + ERL arm throughout treatment, indicating that the increased levels of total cfDNA in the RAM + ERL arm were likely due to increased tumor cell apoptosis of cells other than *EGFR* mutation-positive cells. This phenomenon suggests that RAM may promote continuous tumor cell apoptosis throughout treatment with RAM + ERL (Figure S4). However, further investigation is required for the clinical application of monitoring the size of cfDNA.

Although longitudinal evaluation of mutations was pre-specified, this analysis was exploratory and intended to be hypothesis generating. Nevertheless, our results suggest that RAM enhances the sustained anti-tumor effect of ERL on *EGFR* mutation-positive tumors. We hypothesize 2 scientific reasons behind this result. The first is a direct effect on the tumor by dual blockade of EGFR and VEGF. Tumor cells express VEGFR2 (38), and the EGFR and VEGF pathways are interconnected (39). Furthermore, VEGFR2 inhibition also has a direct anti-tumor effect on cancer cells. In xenograft mouse models of *EGFR*-, *ALK*-, or *ROS1*-altered NSCLC, the combination of VEGFR2 blockade with molecular targeted agents showed enhanced anti-tumor effects of the molecular targeted agents (40). Thus, blockade of VEGFR2 with RAM may similarly enhance the anti-tumor effect of EGFR blockade by ERL. The second scientific reason that we hypothesize for the enhanced anti-tumor effect of ERL on *EGFR*-mutated cells by RAM is an indirect effect on the tumor by inhibition of angiogenesis by RAM. Inhibition of angiogenesis normalizes tumor vessels (41,42), and the improved vasculature enhances drug (ERL) delivery to the tumor (43,44), thereby increasing its efficacy. ERL increases the apoptosis of *EGFR* mutation-positive tumor cells (45), thus reducing the count of *EGFR*-activating mutation alleles.

In this study, we observed that the count of *EGFR*-activating mutation alleles was suppressed throughout treatment in both treatment arms (ERL effect). However, despite this suppression, most patients will progress, indicating that the tumor eventually becomes treatment resistant. One reason for this may be intra-tumoral heterogeneity arising from genetic and epigenetic alterations derived from genomic and chromosomal instability and different patterns of clonal evolution; such heterogeneity has been reported in NSCLC (46,47). Furthermore, in this study, we observed short fragment-sized cfDNA increased in the RAM + ERL arm but not the PL + ERL arm. We hypothesize that the increased concentration of short fragment-sized cfDNA observed with RAM + ERL was derived from tumor cells other than *EGFR* mutation-positive cells; this is supported by observations in a previous study, whereby inhibition of angiogenesis with an anti-VEGFR2 antibody (DC101) led to an increase in apoptosis of endothelial cells followed by apoptosis of tumor cells (48,49), which would result in a release of fragmented DNA. Indeed, RAM monotherapy has been shown to have survival benefits in patients with advanced gastric or

gastroesophageal junction adenocarcinoma progressing after first-line chemotherapy, presumedly by targeting the VEGF pathway and inhibiting angiogenesis (50). This additional anti-tumor effect of RAM by inhibiting VEGFR2 on both tumor and non-tumor cells may contribute to the PFS benefit observed in the RELAY study with RAM + ERL compared with PL + ERL.

This study used both ddPCR and NGS methodologies to identify potential biomarkers in liquid biopsy samples, which, for most analyses, were taken at multiple time points throughout treatment and at post-study treatment discontinuation follow-up. NGS or an electrophoretic mobility assay such as the Bioanalyzer can be used to determine the proportion of ctDNA in a sample (51). In this exploratory study, the Bioanalyzer was used to compare the size distribution of cfDNA. The Bioanalyzer has the advantage of high sensitivity (down to 5 pg/μL for fragment analysis) and requires a smaller sample volume (1 μL for nucleic acids). However, the study was limited to patients with both baseline and 30-day follow-up samples, thus limiting the sample size of the study and making it difficult to draw inferences regarding treatment-emergent mutations. Furthermore, the current data may be biased toward patients who discontinued study treatment, some of whom were early progressors, because patients who were still on treatment were not included.

Conclusions

In conclusion, this biomarker analysis indicated that the count of *EGFR*-activating mutation alleles was suppressed, total cfDNA concentration was increased, and short fragment-sized cfDNA increased with RAM+ERL. Taken together, these results suggest that the additional anti-tumor effect of RAM by inhibiting VEGFR2 on tumor and non-tumor cells may contribute to the PFS benefit observed in the RELAY study with RAM + ERL compared with PL + ERL.

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Footnote

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Trial Protocol: Available in the Supplementary Appendix of Nakagawa K, Garon EB, Seto T, *et al.* Ramucirumab plus erlotinib in patients with untreated, EGFR-mutated, advanced non-small-cell lung cancer (RELAY): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2019;20(12):1655-69. doi:10.1016/S1470-2045(19)30634-5.

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-736/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), the Council for International Organizations of Medical Sciences International Ethical Guidelines, Good Clinical Practice guidelines, and local guidelines. The study and study addendum were approved by the ethics review boards at each site (details are provided in Table S1). Written informed consent was obtained from all individual participants. Patients who opted to participate in the exploratory liquid biopsy addendum provided additional consent.

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