EDITORIAL

Plasma Genotyping From the CROWN, ALTA-1L, and ALEX Trials: Can We Speak With One Voice on What to Test, How to Test, When to Test, and for What Purpose?

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As we continue to advance oncology patient care in this golden era of targeted therapy, integrating plasma genotyping into optimal treatment and trial design is the missing "holy grail." Initially, plasma genotyping was primarily used as an alternative for tumor rebiopsy to identify resistance mechanisms owing to ease of use, faster turnover time, and rebiopsy that may be technically difficult or unsafe to patients. In addition, current plasma genotyping in targeted therapy of NSCLC with actionable driver mutation is being used for disease prognostication at initial diagnosis and for early response monitoring after treatment initiation. There are many ways to perform plasma genotyping, including analysis of cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating tumor cells, or just identifying the specific driver-specific founder mutation.[1]

In *EGFR*-mutated NSCLC, the detection of the underlying *EGFR* mutation in plasma defines a positive ctDNA regardless of the assay (reverse transcription–polymerase chain reaction, droplet polymerase chain reaction, next-generation sequencing). The ability to detect *EGFR* mutations in plasma before treatment in AURA3 and FLAURA patients identified a poor prognostic group with a shorter median progression-free survival (PFS) regardless of treatment with osimertinib or comparator arm (gefitinib/erlotinib or platinum-based chemotherapy respectively), likely because of higher tumor burden.[2,3] Furthermore, the clearance of ctDNA at 3 and 6 weeks after treatment with osimertinib portend a significantly longer PFS.[4]

On the contrary, the detection of ereptory tyrosine kinase fusion positive (RTK+) NSCLC such as *ALK, ROS1,* and *RET* fusions by plasma genotyping is more complex. Ideally, DNA primers span large regions of intronic and exon breakpoints to detect as many fusion variants as possible.[5] Hence, the sensitivity of plasma genotyping on the basis of DNA sequencing varies by the sequencing platform. Plasma RNA NGS should be the next-level plasma genotyping to detect in-frame RTK fusion transcripts, but the technology is not commercially available yet. Hence, the conventional definition of ctDNA in solid tumor oncology with nonspecific oncogenic and tumor suppressor gene mutations such as *TP53, ARIAD1A, TERT* promoter with or without concurrent *ALK* fusion detection has been applied to major pivotal studies of *ALK*-positive (*ALK*+) NSCLC[6–9] (Table 1). However, the definition of positive plasma genotyping by RTK fusion, nonspecific tumor DNA, or a combination of both is confusing and challenging to apply in daily clinical settings owing to the difference in sequencing platforms especially differences in the number of genes sequenced and continuously subjected to technology obsolescence. Second, and less often appreciated, is the detection methods (fluorescence in situ hybridization or immunohistochemistry [IHC]) of *ALK* fusions have potential false-positive rates ranging from 10% to 20% inferred from pivotal randomized phase 3 clinical trials (PROFILE1014, ALEX, and CROWN).[10–12]

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Table 1. Comparison of the Plasma Genotyping Analysis and Main Outcomes of the Three Global Pivotal ALK TKI Trials

Trial	CROWN (n = 293)[9,12]		ALTA-1L (n = 275)[8]	ALEX (n = 303)[6,7]
Authors	Soo et al.[9]	Bearz et al.[12]	Camidge et al.[8]	Dziadziuszko et al.[6] Camidge et al.[7]
ALK TKI (with plasma samples)	Lorlatinib (n = 134)		Brigatinib (n = 123)	Alectinib (n = 137)
Comparator (with plasma samples)	Crizotinib (n = 129)		Crizotinib (n = 127)	Crizotinib (n = 139)
NGS platform	Guardant360 v2.11 (Guardant Health) (plasma) Guardant360 v2.13 (Guardant Health) (tissue)		ctDx Lung NGS panel (Resolution Bioscience, Kirkland, WA)	FoundationACT (Foundation Medicine Inc.)
Genes analyzed (n)	74		38	62 (6 fusions)
Time points analyzed	Baseline, week 4, week 24, EOT	Baseline	Baseline, cycle 3 and d 2, EOT	Baseline
Purpose of test	1. ctDNA as a prognostic biomarker 2. ctDNA as a response monitor	1. ctDNA as a prognostic biomarker 2. *EML4-ALK* variants and *TP53* mutations as biomarkers for ORR and PFS	Prognostic biomarker, monitoring tool; *EML4-ALK* variants and *TP53* mutations as biomarkers for ORR and PFS	1. Bassline median cfDNA, ctDNA, *EML4-ALK* variants, and *TP53* mutations as biomarkers for ORR and PFS.
Plasma genotyping	ctDNA	*ALK* fusions, TP53	*ALK* fusion, *TP53*	cfDNA, ctDNA, *ALK* fusions, *TP53*
Plasma samples available for analysis (% of total ITT patients enrolled)[a]	90.4% (263/291) 90.4% (263/291)		90.9% (250/275)	91.1% (276/303)
Percentage of plasma genotyping successfully performed/evaluable	96.6% (254/263)		100% (250/250)	80.4% (222/276)

(continued)

Table 1. Continued

Trial	CROWN (n = 293)[9,12]			ALTA-1L (n = 275)[8]	ALEX (n = 303)[6,7]
Percentage of plasma genotyping positive for ctDNA or cfDNA	Analysis of on-treatment ctDNA dynamics 66.2% (174/263) Lorlatinib (n = 96) Crizotinib (n = 78)		78.3% (206/263) Lorlatinib (n = 102) Crizotinib (n = 104)	NA	79.3% (176/222) Alectinib (n = 87); Crizotinib (n = 89)
VAF cutoff as positivity		>0.3%		>0.1%	≥ 0.5%
Biomarker analysis at baseline					
Median cfDNA amount		NR		not performed	11.53 ng/mL
Definition of plasma genotyping positivity	All tumor genomic ctDNA		*ALK* fusion, *TP53* mutation	*ALK* fusion, *TP53* mutation	median cfDNA
Percentage of *ALK* fusion variants detected among successful genotyping	NA		64.0% (126/197) Lorlatinib (n = 62), Crizotinib (n = 64)	54.0% (136/250) Brigatinib (n = 67) Crizotinib (n = 69)	Plasma: 65% (145/222) Tumor: 67% (136/203)
Percentage of positive *TP53* mutation	NA		41.6% (82/197) Lorlatinib (n = 41), Crizotinib (n = 41)	33.1% (45/136) *TP53* mutation only reported in *ALK* fusion-positive samples: Brigatinib (n = 22), Crizotinib (n = 23)	32.4% (72/222) Alectinib (n = 40), Crizotinib (n = 32)
Biomarkers correlated with clinical response	dVAF, molecular responders, molecular response at 4 weeks (NCTD, MRC, MRNC, MNR)		*EML4-ALK* fusion variant, *TP53* mutation	*EML4-ALK* fusion variant, *TP53* mutation	≤median cfDNA vs. >median cfDNA
Clinical efficacy					
Surrogate marker of tumor burden	Yes		NR	NR	Yes positive correlation for no. of lesions, no. of organ sites involved, tumor size, presence of brain metastasis, *TP53* mutation

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Table 1. Continued

Trial	CROWN (n = 293)[9,12]		ALTA-1L (n = 275)[8]	ALEX (n = 303)[6,7]
ctDNA or cfDNA	12-month PFS rate by changes in baseline VAF at wk4 (≤ vs. >baseline) Lorlatinib ≤VAF: 83% (71-90) Lorlatinib > VAF: 69% (46-84) HR = 0.50 (0.23 - 1.1) Crizotinib ≤ VAF: 31% (20-43) Crizotinib > VAF: 21% (3-48) HR = 1.00 (0.49-2.03) 12-month PFS rate by no detectable ctDNA and response categories at wk 4 molecular responders [MR] (ratio <50% of mean VAF) vs. nonresponders [MNR] (ratio >50%) Lorlatinib no ctDNA detected: 89% (61-97) MRC: 86% (72-93) MRNC: 75% (41-91) MNR: 56% (29-76) Crizotinib no ctDNA detected: 79% (53-92) MRC: 32% (18-49) MRNC: 9% (1-33) MNR: 38% (12-64)	NR	NR	Median PFS by median cfDNA Alectinib ≤ median cfDNA: NR (34.9-NR) Alectinib > median cfDNA: 14.8m (10.9-40.4) Crizotinib ≤ median cfDNA: 14.8m (12.7-25.6) Crizotinib > median cfDNA: 8.6m (7.2-10.8) Median PFS by ctDNA positivity/negativity Alecitinib ctDNA-: NE (95% CI: NE -NE) Alectinib ctDNA+: 22.4m (95% CI: 14.6 -NE) Crizotinib ctDNA-: 16.6m (95% CI: 10.8- 27.6) Crizotinib ctDNA+: 8.8m (95% CI: 7.3-10.8)
EML-ALK4 variants (95% CI)	NR	ORR by lorlatinib: v1 (80%; 56.3-94.3), v2 (85.7%; 42.1-99.6), v3 (72.2%; 46.5-90.3) ORR by crizotinib: v1 (50%; 29.9-70.1), v2 (50%; 1.3-98.7), v3 (73.9%; 56.1-89.8) Median PFS by lorlatinib: v1 (NR; NR-NR), v2 (NR; 32.9-NR), v3 (33.3m; 14.7-NR) Median PFS by crizotinib: v1 (7.4m; 5.5-9.3), v2 (NR; 3.7-NR), v3 (5.5m; 5.3-9.2)	ORR by brigatinib: v1 (84%), v2 (83%), v3 (83%) ORR by crizotinib: v1 (73%), v2 (60%), v3 (67%) Median PFS by brigatinib: v1 (29.0m), v2 (16.0m), v3 (16.0m) Median PFS by crizotinib: v1 (13.0m), v2 (11.0m), v3 (7.4m)	ORR by alectinib: v1 (90.5%), v2 (70.0%), v3 (83.3%). ORR by crizotinib: v1 (66.7%), v2 (63.6%), v3 (45.8%) Median PFS by alectinib: v1 (34.8m), v2 (24.8m), v3a/b (17.7m) Median PFS by crizotinib: v1 (7.4m), v2 (8.8m), v3a/b (9.1m)

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Table 1. Continued

Trial	CROWN (n = 293)[9,12]		ALTA-1L (n = 275)[8]	ALEX (n = 303)[6,7]
TP53mt vs. *TP53wt* (95% CI)	NR	Plasma genotyping: ORR by lorlatinib: *TP53wt*: 78.6% (65.6-88.4) *TP53mt*: 70.7% (54.5-83.9) Median PFS by lorlatinib: *TP53wt*: NR (NR-NR) *TP53mt*: NR (18.2-NR) (HR = 0.65, 0.333-1.261) ORR by crizotinib: *TP53wt*: 61.0m (47.4-73.5) *TP53mt*: 56.1m (39.7-71.5) Median PFS by crizotinib: *TP53wt*: 10.8m (7.4-11.4) *TP3mt:* 7.2m (5.4-9.3) (HR = 0.71, 0.437-1.151) Tumor tissue: ORR by lorlatinib: *v1/TP53wt*: 86.7% (59.5-98.3) *v1/TP53mt*: 83.3% (35.9-99.6) *v3/TP53wt*: 77.8% (52.4-93.6) *v3/TP53mt:* 85.7% (42.1-99.6) Median PFS by lorlatinib: *v1/TP53wt:* NR (25.8-NR) *v1/TP53mt:* NR (11.0-NR) *v3/TP53wt:* NR(NR-NR) *v3/TP53mt:* NR (NR-NR) ORR by crizotinib: *v1/TP53wt:* 50.0% (24.7-75.3) *v1/TP53mt:* 33.3% (0.8-90.6) *v3/TP53wt:* 83.3% (51.6-97.9) *v3/TP53mt:* 50.0% (18.7-81.3) Median PFS by crizotinib: *v1/TP53wt:* 12.9m (7.4-NR) *v1/TP53mt:* 15.6m (9.2-21.9) *v3/TP53wt:* 10.9m (5.3-14.8) *v3/TP53mt:* 6.7m (3.7-9.3)	ORR by brigatinib: *EML4-ALK/TP53wt*: 89% (73-97) *EML4-ALK/TP53mt*: 77% (55-92) Median PFS by brigatinib: *EML4-ALK/TP53wt*: 24.0m (18.0-NR) *EML4-ALK/TP53mt*: 18.0m (5.6-NR) ORR by crizotinib: *EML4-ALK/TP53wt*: 71% (55-84) *EML4-ALK/TP53mt*: 61% (39-80) Median PFS by crizotinib: *EML4-ALK/TP53wt*: 13.0m (9.2-21.0) *EML4-ALK/TP53mt*: 7.4m (5.6-13.0)	Median PFS (univariate analysis): *TP53mt* vs. *TP53wt* (HR = 2.42; 95% CI: 1.36-4.32; p = 0.0027)

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Table 1. Continued

Trial	CROWN (n = 293)[9,12]		ALTA-1L (n = 275)[8]	ALEX (n = 303)[6,7]
Preexisting bypass pathway resistance alteration[b]	NR	14.2% (28/197) Lorlatinib (n = 15), Crizotinib (n = 13) ORR by lorlatinib: Mutation positive: 73.3 (44.9–92.2) Mutation negative: 75.6 (64.9–84.4) Median PFS by lorlatinib: Mutation positive: NR (20.2–NR) Mutation negative: NR (32.9–NR) ORR by crizotinib: Mutation positive: 30.8 (9.1–61.4) Mutation negative: 63.2 (52.2–73.3) Median PFS by crizotinib: Mutation positive: 10.3 (2.2–NR) Mutation negative: 8.5 (7.2–10.9)	NR	NR
Acquired *ALK* mutation at EOT	Lorlatinib (0%) vs. Crizotinib (10%)[16]		Brigatinib (n = 2), Crizotinib (n = 10)	NA as only baseline cfDNA was collected

[a]Samples from patients enrolled in the People's Republic of China were not available for plasma genotyping.
[b]Included canonical alteration in the MAPK/PI3K pathway such as the following: (1) mutation and or amplification of *BRAF*, *KRAS*, *PIK3CA*, (2) alterations in receptor tyrosine kinase family of receptors (*EGFR*, *ERBB2*, *KIT*, *MET* mutation/amplification), or (3) cell cycle regulation such as *CDK4*, *CDK6*, *CCND1*, *CCND2*, *CCNE1* amplification, or *CDKN2A* or *RB1* mutation.
cfDNA, cell-free DNA; CI, confidence interval; ctDNA, circulating tumor DNA; dVAF, change in VAF; EOT, end of treatment; HR, hazard ratio; ITT, intention to treat; MNR, molecular nonresponder; MRC, molecular responder cleared; MRNC, molecular responder not cleared; mt, mutant; n, number; no., number; NA; not available; NCTD, no ctDNA detected; NGS, next-generation sequencing; no., number; NR, not reported; PFS, progression-free survival; TKI, tyrosine kinase inhibitor; v1, variant 1; v2, variant 2; v3, variant 3; VAF, variant-allele frequency; wt, wild-type.

In this issue of the Journal of Thoracic Oncology, both Soo et al.[9] and Bearz et al.[12] reported on complementary aspects of plasma genotyping in the CROWN trial. Soo et al.[9] reported that both the absolute changes (allele frequency) and relative changes (percentage changes with references to mean allele frequency of the whole patient group, which is not easily applicable to daily practice to reference the "group" mean since the real world *ALK*+ NSCLC patients may not represent the CROWN "group" of patients) changes in ctDNA level corresponds to PFS achieved by lorlatinib and crizotinib. In addition, there is a positive correlation between tumor burden and detection of ctDNA as previously reported for *EGFR-positive* NSCLC.[2,3] Previously, ALEX investigators reported that the higher the amount of pretreatment cfDNA, the less favorable the treatment outcome.[6]

Most importantly, Soo et al.[9] reported that initial ctDNA-negative patients (no ctDNA detected [NCTD] group) achieved the best PFS treatment outcome regardless of treatment with either lorlatinib or crizotinib. The complete clearance of ctDNA (molecular response–cleared group) at the first follow-up exhibited the second best favorable PFS when compared with in ctDNA-negative lorlatinib-treated patients. Similarly important for hypothesis generation, lorlatinib-treated patients that did not achieve any molecular response at week 4 (molecular nonresponse group, N = 8) only achieved a median PFS of 14.7 months (95% confidence interval [CI]: 2.5–not reached). This molecular nonresponse subgroup needs further molecular characterization and may benefit from additional combination treatment such as the addition of chemotherapy. To our knowledge, Soo et al.[9] are the first to report the clearance of ctDNA in *ALK*+ NSCLC results in prognostic and predictive significance (Table 1). In crizotinib-treated patients in CROWN, the initial ctDNA-negative patient group achieved numerically the longest PFS of 18.5 months (95% CI: 14.8–not reached), albeit still significantly shorter than lorlatinib-treated NCTD group.[9] However, both non-detectable ctDNA at baseline or the ability to clear ctDNA in the crizotinib-treated patient group did not have statistically significant improvement in PFS when compared with crizotinib-treated patients with persistent detectable ctDNA.

Bearz et al.[12] reported the prognostic significance of *EML4-ALK* variants (variant 1 [v1], variant 2 [v2], and variant 3 [v3]) and *TP53* mutations alone or in combination on the basis of both tumor and plasma profiling identified before treatment. In agreement with ALEX and ALTA-1L results, *EML4-ALK* v3[7,8] or the presence of *TP53* mutations from plasma genotyping[6,8] negatively modulated the PFS achieved by next-generation ALK TKIs (Table 1). Notably, *TP53* mutations regardless of whether considered "pathogenic" or "nonpathogenic" resulted in shortened PFS in lorlatinib-treated patients.[12] More importantly, Bearz et al.[12] are the first to report in a randomized phase 3 clinical trial that the combination of *EML4-ALK* and the presence of *TP53* mutations (N = 25) had the shortest median PFS of 20.2 months (95%CI: 14.8–not reached) even when treated with lorlatinib.

Whereas all three trials came to a similar conclusion, the subgroup analysis of plasma genotyping was different. It remains a challenge to distinguish cfDNA from ctDNA in the ALEX trial. The ctDNA definition varies by the commercial test used and sequencing platforms change over time. The FoundationACT (Foundation Medicine, Cambridge, MA) (62 genes) used to identify ctDNA in ALEX has now been replaced by Foundation ONE CDx (Foundation Medicine) (324 genes) commercially so any clinical laboratories are unlikely to go back in time to use the 62 gene panel so they can interpret the ALEX data. Similarly, a newer version of Guardant360 (GuardantHealth, Redwood City, CA) (83 genes) is being offered along the Guardant360CDx (74 genes), the platform used for the CROWN ctDNA analysis. Thus, the "timelessness" of the reported cfDNA and ctDNA sequencing platforms is short-lived, and the results of ALEX and CROWN cannot be reproduced using current commercial platforms. The Resolution Bioscience (now part of Agilent) ctDNA (38 genes) test used in ALTA-1L is not widely used commercially in the US.

Low tumor burden leads to inadequate tumor DNA shedding and nondetectable ctDNA, which portends a good prognosis. The lower sensitivity of DNA-based NGS plasma genotyping to detect all *ALK* rearrangements is, potentially, an explanation for the 55% to 65% detection rate of *ALK* fusions by plasma genotyping across all three trials (Table 1). Furthermore, in the CROWN trial reported by Bearz et al.,[12] *ALK* fusions were detected only in 80% of the tumor samples, implying a potential false-positive rate of 20%.[12] ALK-positivity by Ventana IHC system (Roche, Rotkreuz, Switzerland) performed at local laboratories (without central review) was the *ALK* detection entry criteria into CROWN. The treatment efficacy of these 20% of patients (IHC+, NGS-) by loratinib and crizotinib was not reported. In the ALEX study, the false-positive rate of ALK IHC with a central review (with ALK fluorescent in situ hybridization as the accepted standard) was approximately 13% with a shorter and non-significant PFS among the potentially false-positive group.[11] Thus, potentially a portion of the negative ctDNA results could result from an initial false-positive IHC test. In the near future, the use of next-generation targeted RNA sequencing or whole transcriptome sequencing in tumor or plasma genotyping should

increase the sensitivity and specificity of *ALK* fusion variants detection, thereby identifying favorable prognostic groups that are truly negative by plasma genotyping. Furthermore, next-generation RNA sequencing may provide better snapshots of the dynamic ratios of different splice variants of *EML4-ALK* 3a/3b, which have predictive significance to response to ALK TKIs and treatment implication and optimization in *ALK*+ NSCLC.[13,14]

All three trials had baseline plasma genotyping before treatment, which was generally predictive of outcome. In the CROWN report by Soo et al.,[9] these ctDNA-negative patients had the best outcome treated within crizotinib-treated or lorlatinib-treated arm, a finding consistent with the concept of low tumor burden resulting in better prognosis regardless of treatment although better trreatment still resulted in longer PFS. In all three trials, *EML4-ALK* variants and *TP53* mutations were prognostic for PFS regardless of next-generation *ALK* TKI. More importantly, knowing whether a patient sheds tumor DNA such as *EML4-ALK* variants (or other *ALK* fusion partners), and *TP53* mutations status before treatment will serve as a useful reference in interpreting future resistance mechanisms, especially when *ALK* fusion is not detected at the time of progression.

The optimal time point for early ctDNA dynamics for treatment monitoring of *ALK*+ NSCLC remains to be elucidated. Soo et al.[9] assessed early ctDNA dynamics during week 4 and week 24, and deeper analysis of molecular response at week 4 was subdivided by molecular responders and nonresponders, which revealed that clearance of ctDNA is important for predicting improved PFS outcome, especially among lorlatinib-treated patients.[9] The other two pivotal trials (ALEX, ALTA-1L) did not have a 4-week plasma genotyping monitoring requirement within the protocol. Hence, ctDNA treatment dynamics early on likely predicts eventual treatment outcomes. Left undiscussd by Soo et al.[9] is the disposition of the patients who were molecular non-responders which accounted for 17% of the ctDNA positive patients treated on the lorlatinib arm whether to continue lorlatinib or more likely additional treatment (i.e. chemotherapy) should be added to lorlatinib early on in the treatment course.

Still currently plasma genotyping is used sparingly and usually obtained at the time of progression for future treatment decisions during treatment of *ALK*+ NSCLC understandably due to the cost and the questions underpinning this editorial. As alluded to earlier, prognostication and monitoring early response using plasma genotyping may guide treatment options in the future. In *ALK*+ NSCLC, detecting the presence of *EML4-ALK* v3 and knowledge of *TP53* mutation status are important investigational uses of plasma genotyping. Given that Bearz et al.[12] reported that *EML4-ALK/TP53mt ALK*+ NSCLC had the worst PFS among lorlatinib-treated patients, future clinical trials should consider stratifying both *EML4-ALK* variants (v1, v3, others) and *TP53* status (wild-type versus mutant).[12] The just published ALTA-3 randomized trial pointed to the need to stratify for the three important factors discussed in this editorial: presence/absence of ctDNA, *EML4-ALK* v1/3, and *TP53* wild-type/mutated each has important implication on the success or failure of randomized *ALK*+ NSCLC trials[15] and should be considered as stratification factors TKIs going forward in the development of next-generation ALK.

CRediT Authorship Contribution Statement

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