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ORIGINAL ARTICLE

Clinical utility of plasma-based digital next-generation sequencing in patients with advance-stage lung adenocarcinomas with insufficient tumor samples for tissue genotyping

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Background: Approximately 30% of tumor biopsies from patients with advanced-stage lung adenocarcinomas yield insufficient tissue for successful molecular subtyping. We have analyzed the clinical utility of next-generation sequencing (NGS) of cell-free circulating tumor DNA (ctDNA) in patients with inadequate tumor samples for tissue genotyping.

Patients and methods: We conducted the study in a multi-institutional prospective cohort of clinically unselected patients with advanced-stage lung adenocarcinomas with insufficient tissue for *EGFR*, *ALK* or *ROS1* genotyping across 12 Spanish institutions ($n = 93$). ctDNA NGS was carried out by Guardant Health (Guardant360, Redwood City, CA), using a hybrid-capture-based 73-gene panel. Variants were deemed actionable if they were part of the OncoKB precision oncology knowledge database and classified in four levels of actionability based on their clinical or preclinical evidence for drug response.

Results: Eighty-three out of 93 patients (89%) had detectable levels of ctDNA. Potentially actionable level 1–4 genomic alterations were detected in 53 cases (57%), of which 13 (14%) had level 1–2A alterations (Food and Drug Administration-approved and standard-care biomarkers according to lung cancer guidelines). Frequencies of each genomic alteration in ctDNA were consistent with those observed in unselected pulmonary adenocarcinomas. The majority of the patients (62%), particularly those with actionable alterations (87%), had more than one pathogenic variant in ctDNA. The median turnaround time to genomic results was 13 days. Twelve patients (13%) received genotype-matched therapies based on ctDNA results, deriving the expected clinical benefit. Patients with co-occurring pathogenic alterations had a significantly shorter median overall survival as compared with patients without co-occurring pathogenic alteration (multivariate hazard ratio = 5.35, $P = 0.01$).

Conclusion: Digital NGS of ctDNA in lung cancers with insufficient tumor samples for tissue sequencing detects actionable variants that frequently co-occur with other potentially clinically relevant genomic alterations, allowing timely initiation of genotype-matched therapies.

Key words: lung adenocarcinoma, insufficient tissue, ctDNA, digital next-generation sequencing, actionable genomic alterations, co-occurring genomic alterations

Introduction

Tissue genotyping is standard of care in the treatment algorithm of patients with advanced-stage, non-squamous, non-small-cell lung cancers (NSCLCs) [1]. As the actionable drivers continue to expand beyond *EGFR*, *ALK* and *ROS1*, multiplexed tissue-based genotyping algorithms, including targeted next-generation sequencing (NGS) panels, have increasingly become routine practice in many institutions. However, even using tissue-sparing or more efficient NGS protocols, approximately 30% of tumor samples still yield insufficient or inadequate tissue for successful molecular genotyping [2, 3]. This tissue failure rate can be even higher when using multiplexed single-gene tests or sequential sequencing protocols [4]. Repeated biopsies are not feasible in many of these patients, because of anatomical difficulties, existing comorbidities and/or because some may suffer from clinical deterioration that forces rapid initiation of medical treatment. In this clinical context, the use of robustly validated plasma-based NGS panels could be particularly useful, as they can detect actionable alterations in cell-free circulating tumor DNA (ctDNA) with sufficient sensitivity and specificity to avoid repeated tissue biopsies [5].

In the present study, we have carried out targeted ctDNA NGS in a multi-institutional prospective cohort of patients diagnosed with advanced-stage lung adenocarcinomas with insufficient or unavailable tumor samples for tissue sequencing. Our primary objective was to demonstrate the clinical utility of plasma-based NGS to select these patients for genotype-tailored therapies.

Methods

Institutional Ethics Committee approval was obtained before this study was initiated. A single protocol was contemporaneously distributed across 12 participating Spanish institutions. Consecutive patients with advanced-stage lung adenocarcinomas with insufficient or inadequate tumor samples for standard care *EGFR*, *ALK* or *ROS1* genotyping [1] were eligible for the study. Repeated tumor biopsies had to be considered technically or clinically unfeasible by practicing physicians. Patients with already known genomic alterations in *EGFR*, *ALK* or *ROS1* were not eligible for plasma sequencing in this cohort. All patients provided signed informed consents before plasma genotyping and were subsequently registered in the study.

We obtained blood samples from patients and ctDNA was isolated from plasma. Digital NGS of ctDNA was carried out by Guardant Health, Inc. (Guardant360, Redwood City, CA), a Clinical Laboratory Improvement Amendment-certified, College of American Pathologists-accredited and New York State Department of Health-approved clinical laboratory. A targeted, hybrid-capture-based, NGS panel detecting all four major types of genetic alterations in 73 genes (supplementary Figure S1, available at *Annals of Oncology* online) was used in all patients. Detailed protocols for ctDNA isolation, sequencing and data analysis have been described elsewhere [6] (supplementary Methods, available at *Annals of Oncology* online).

We classified genomic variants detected in each plasma sample in two main categories based on their functional and biological relevance: (i) Variants of unknown significance, those genomic alterations whose functional consequences and clinical significance has not been established; and (ii) Pathogenic or deleterious variants, those genomic alterations validated or predicted to affect gene function. These deleterious variants were then subclassified according to their therapeutic vulnerability in actionable and non-actionable alterations. We considered deleterious variants as therapeutically actionable alterations if they were part of the OncoKB precision oncology knowledge database, and we annotated them in four levels of actionability based on their clinical or preclinical evidence for drug response [7, 8]: level 1, Food and Drug Administration-approved biomarkers in patients with lung cancer (e.g. *EGFR* exon 19 deletions); level 2A, standard of care biomarkers for approved drugs based on lung cancer clinical practice guidelines (e.g. *MET* exon 14 alterations); level 2B, approved biomarkers in another cancer indication but not in patients with lung cancer (e.g. *HER2* amplifications); level 3, alterations with promising clinical evidence for drug response but not currently standard of care in any cancer type (e.g. *PIK3CA* mutations); level 4, alterations with compelling preclinical evidence of drug response (e.g. *KRAS* mutations). In the case of copy number gains in actionable genes, we considered any level of reported amplification [reportable range $\geq$2.12 copies (supplementary Methods, available at *Annals of Oncology* online)] as therapeutically vulnerable, with the exception of *MET* copy number gains, which only high-level amplifications [reported as 3+ amplifications (supplementary Methods, available at *Annals of Oncology* online)] were annotated as actionable [9]. Patients with more than one actionable variant were annotated with their highest level of actionable alteration [8]. On the other hand, we classified pathogenic variants as non-actionable alterations if they were not considered to be therapeutically vulnerable by OncoKB (e.g. *TP53* or *STK11* mutations) [7].

We used *t*-test or one-way analysis of variance to study the association between continuous variables, and chi-square test and binary logistic regression to analyze the association between categorical variables. Overall survival (OS) and progression-free survival were defined as the time interval between the date of blood sample extraction and the date of death or loss of follow-up, and the date of initiation of genotype-tailored therapies and the date of disease progression or loss of follow-up, respectively. For those patients who did not die/progress during the study period, the outcome was considered left-censored. Survival curves were estimated with the Kaplan–Meier product-limit method and compared using the log-rank test. Hazard ratios (HRs) for OS were calculated using the Cox regression model. All hypothesis testing was carried out at a two-sided significance level of $\alpha = 0.05$.

Results

We included a total of 93 patients, 48 (52%) of which were treatment naive and 51 (55%) had extra-thoracic metastasis. The median age was 63 years (range 33–89), and 24 patients (26%) were never smokers. Fifty-six (60%) patients had insufficient tumor tissue to carry out any genomic molecular test (Table 1).

Eighty-three patients (89%) had detectable genomic variants in ctDNA (including variants of unknown significance).

Table 1. Baseline characteristics of the patients

Characteristic	*N* (%)	ctDNA positive (%)	ctDNA negative (%)	*P* value
Total	93	83 (89)	10 (11)	
Gender				0.35
Male	43 (46)	37 (45)	6 (60)	
Female	50 (54)	46 (55)	4 (40)	
Age (years)				0.21
<65	48 (52)	41 (49)	7 (70)	
≥65	45 (48)	42 (51)	3 (30)	
Smoking history				0.57
Never smoker	24 (26)	21 (25)	3 (30)	
Former smoker	42 (45)	39 (47)	3 (30)	
Current smoker	27 (29)	23 (28)	4 (40)	
Performance status				0.53
0	34 (37)	31 (37)	3 (30)	
1	48 (52)	41 (49)	7 (70)	
2	8 (8)	8 (10)	0	
3	3 (3)	3 (4)	0	
Stage				0.42
Locally recurrent or stage III	3 (3)	2 (2)	1 (10)	
M1a	39 (42)	36 (43)	3 (30)	
M1b	4 (4)	3 (4)	1 (10)	
M1c	47 (51)	42 (51)	5 (50)	
Liver metastasis				0.97
Yes	9 (10)	8 (10)	1 (10)	
No	84 (90)	75 (90)	9 (90)	
Brain metastasis				0.70
Yes	13 (14)	12 (14)	1 (10)	
No	80 (86)	71 (86)	9 (90)	
Bone metastasis				0.31
Yes	32 (34)	30 (36)	2 (20)	
No	61 (66)	53 (64)	8 (80)	
Extra-thoracic lymph nodes				0.70
Yes	13 (14)	12 (15)	1 (10)	
No	80 (86)	71 (85)	9 (90)	
Adrenal metastasis				0.93
Yes	10 (11)	9 (11)	1 (10)	
No	83 (89)	74 (89)	9 (90)	
Pleural or pericardial metastasis/effusion				0.80
Yes	16 (17)	14 (17)	2 (20)	
No	77 (83)	69 (83)	8 (80)	
Number of therapies received				0.91
Treatment naive	48 (52)	42 (51)	6 (60)	
1	31 (33)	28 (34)	3 (30)	
≥2	14 (15)	13 (15)	1 (10)	
Tissue mutational status				
EGFR, *ALK* and *ROS1* unknown	56 (60)			
EGFR wild-type[a]	35 (37)			
ALK wild-type[a]	23 (25)			

[a]Twenty-one tumors were *EGFR* and *ALK* wild-type. *ROS1* was unknown due to insufficient tumor tissue in all cases.

As depicted in Table 1, none of the clinical characteristics analyzed predicted the detection of genomic variants in ctDNA. In the same line, with the exception of female gender [odds ratio (OR) = 2.9, $P = 0.02$], no other clinical factor predicted the detection of pathogenic variants with allele frequencies ≥5% (supplementary Table S1, available at *Annals of Oncology* online).

Seventy-five cases (80%) had at least one pathogenic variant in ctDNA, and level 1–4 actionable alterations were detected in 53

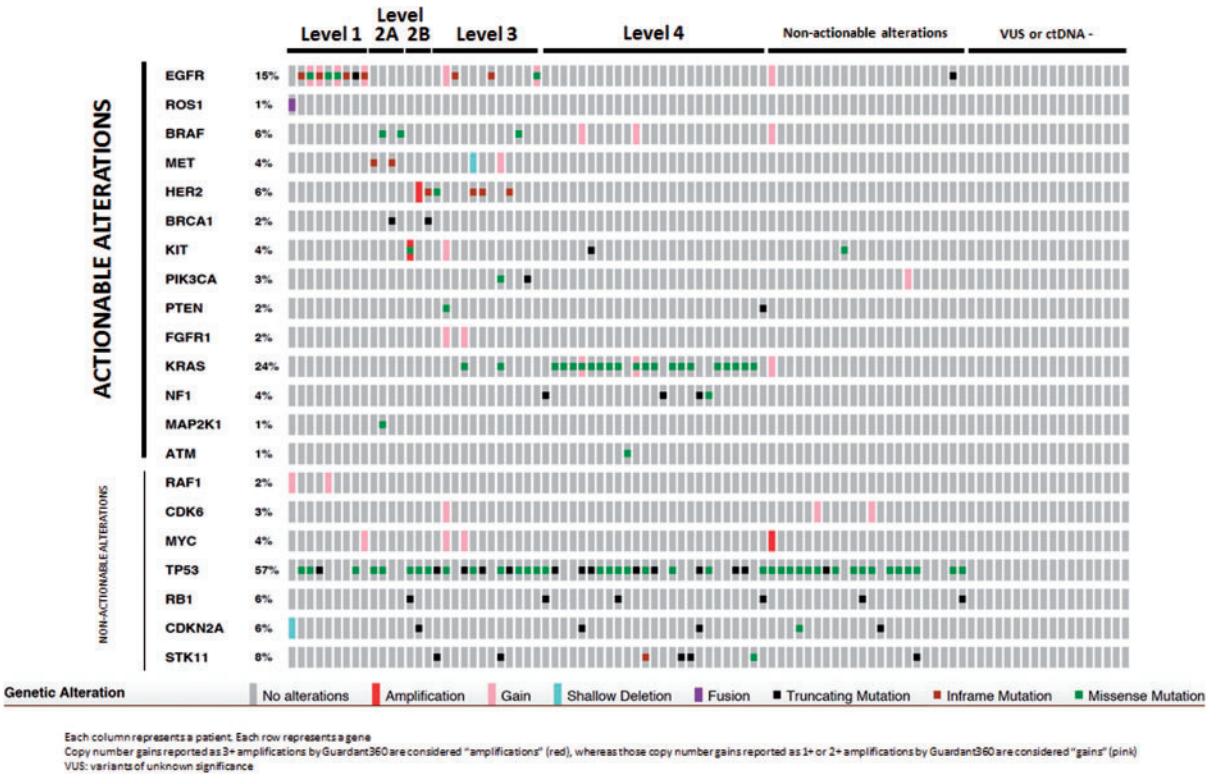

Figure 1. Oncoprint of selected pathogenic alterations detected in ctDNA.

patients (57%), of which 13 (14%) had level 1–2A variants (supplementary Table S2, available at *Annals of Oncology* online). Among the treatment naive patient subgroup, 29 patients (60%) had actionable alterations, and 7 cases (15%) had level 1–2A variants. The presence of extra-thoracic disease was more frequent among patients with actionable alterations detected ($n = 33$, 62%) than among patients with non-actionable alterations detected ($n = 7$, 32%) [OR = 3.53, 95% confidence interval (CI) 1.23–10.15; $P = 0.01$]. The distribution of other clinical factors (age, gender, performance status and smoking history) did not significantly differ between these two groups (supplementary Table S3, available at *Annals of Oncology* online). *KRAS* ($n = 22$, 24%) and *EGFR* ($n = 14$, 15%) were the most frequently observed mutated actionable genes (Figure 1). A complete list of all detected variants per patient with the corresponding allele frequencies and actionable categories can be found in supplementary material, available at *Annals of Oncology* online.

In this cohort, 58 patients (62%) had co-occurring deleterious alterations (i.e. more than one pathogenic variant detected in ctDNA), of which eight patients harbored more than one actionable alteration (Figure 1 and supplementary Table S4, available at *Annals of Oncology* online). Overall, the most frequently detected co-occurring deleterious alterations were pathogenic variants in *TP53* ($n = 48/58$, 83%). Clinical characteristics were similar between patients with co-occurring pathogenic alterations ($n = 58$) and those without co-occurring pathogenic alterations ($n = 35$), except for a non-significantly higher proportion of ever smokers among the subgroup of patients with deleterious co-alterations (81% versus 63%, $P = 0.05$) (supplementary Table S5, available at *Annals of Oncology* online). The vast majority of the patients with level 1–4 actionable variants had co-occurring pathogenic alterations ($n = 46$, 87%), this proportion being significantly lower among the subgroup of patients with non-actionable variants ($n = 12$, 55%) ($P = 0.004$). We found no significant differences in the number of pathogenic alterations per patient in each of the actionable categories (supplementary Figure S2, available at *Annals of Oncology* online).

The median turnaround time from blood extraction to ctDNA results was 13 days (range 7–30). Twelve patients (13%) received effectively matched targeted therapies, mostly in the standard care setting (Table 2). Among the subgroup of treatment naive subjects, six patients (12%) received targeted drugs as their first-line therapy. While most patients with level 1 (89%) and level 2A (50%) alterations received genotype-matched drugs, only a minority of those with level 2B–4 alterations (5%) received targeted therapies (supplementary Table S2, available at *Annals of Oncology* online). The main reason for not receiving therapies matching ctDNA results was either the lack of a clinical trial or inability to access matched drugs outside the context of a clinical study. Three patients, one with a level 1 alteration (uncommon *EGFR* exon 18 deletion) and two with level 4 alterations, suffered from rapid clinical deterioration that precluded their potential drug access.

After a median follow-up of 8 months in the entire population (range 12 days to 15 months), the median OS was not reached in patients who received matched targeted therapies, being 11.5 months in patients who did not receive genotype-matched drugs ($P = 0.32$). The median OS was comparable in patients with detectable (10.4 months) and undetectable (11.5 months) genomic alterations in ctDNA ($P = 0.45$), albeit with a trend toward shorter survival in those with at least one pathogenic variant with an allele frequency $\geq 5\%$ (8.3 versus 12.2 months, $P = 0.09$;

Table 2. Genotype-tailored therapies and their outcomes in patients with actionable alterations in ctDNA

Patient	Highest-level actionable alteration	VAF (%)	Co-mutations	Line of therapy	Treatment	Treatment context	Best response	mPFS (months)	mOS (months)
1	*EGFR* (exon 19 del)	5.5	Yes	First line	Erlotinib ± Ramucirumab	Clinical trial (NCT02411448)	Partial response	*11.8*	*14.1*
2	*EGFR* (exon 19 del)	6.4	Yes	Second line	Afatinib	Standard care	Stable disease	*10.8*	*11.9*
3	*EGFR* (exon 19 del)	11.6	Yes	First line	Afatinib	Standard care	Partial response	*5*	*6.1*
4	*EGFR* (exon 19 del)	0.08	No	First line	Erlotinib	Standard care	Partial response	*7.7*	*11*
5	*EGFR* (L858R)	35.2	Yes	First line	Gefitinib	Standard care	Partial response	*7.2*	*10*
6	*EGFR* (L858R)	0.3	Yes	First line	Gefitinib	Standard care	Not evaluable[a]	*0.7*	1.1
7	*EGFR* (L858R)	10.3	Yes	First line	Afatinib	Standard care	Partial response	*8.1*	*8.2*
8	*ROS1* (SDC4-ROS1)	1.3	Yes	Second line	Crizotinib	Standard care	Partial response	3.6	5.2
9	*BRAF* (V600E)	0.3	No	Forth line	Dabrafenib + trametinib	Compassionate use	Partial response	3.7	*13.4*
10	*MET* (exon 14 skip)	8	Yes	Second line	Crizotinib	Compassionate use	Progressive disease	0.5	1.9
11	*HER2* (S310F)	2.2	Yes	Third line	Paclitaxel + trastuzumab	Compassionate use	Stable disease	2.9	10.4
12	*FGFR1* (AMP)		Yes	Second line	Docetaxel + nintedanib	Standard care	Partial response	2.8	*13.8*

Italicized numbers correspond to censored events.

[a]This patient died of septicemia and the disease could not be evaluated for response.

VAF, variant allele frequency; AMP, amplification; mPFS, median progression-free survival; mOS, median overall survival.

HR 1.68, 95% CI 0.90–3.15) (supplementary Figure S3, available at *Annals of Oncology* online). On the other hand, patients with co-occurring pathogenic alterations had a significantly shorter median OS (8.3 months) as compared with patients without deleterious co-alterations in ctDNA (median not reached; $P = 0.007$) (Figure 2A), with a multivariate HR [adjusted by age, smoking history, presence of extrathoracic metastasis, performance status, variant allele frequency (at least one variant $\geq$5% versus <5%) and matched genotype treatment] of 5.35 (95% CI 1.39–20.47; $P = 0.01$). This shorter survival prediction of the presence of co-occurring deleterious alterations remained significant in the subgroup of patients with actionable variants ($P = 0.03$) (Figure 2B), but not among those with non-actionable variants (5.9 versus 9.1 months; $P = 0.17$) (Figure 2C).

Discussion

In the present study, we show that digital NGS of ctDNA in lung cancers with insufficient tumor samples for tissue sequencing detects actionable variants that frequently co-occur with other potentially clinically relevant genomic alterations and allows timely initiation of genotype-matched therapies. These results complement the findings from other studies using plasma-based NGS in patients with lung cancer [10–12], adding now further evidence to its utility to impact clinical care in a prospective, multicenter and clinically unselected cohort of patients without tumor tissue for molecular analysis.

The Guardant360 NGS technology has been extensively validated in prospective clinical cohorts, showing a high sensitivity for ctDNA mutation detection [6]. A minor proportion of patients in this study had undetectable ctDNA levels. Neither the presence of extrathoracic disease, liver metastasis or bone metastasis, factors that have been shown to increase the sensitivity of other plasma-based sequencing assays [13], predicted ctDNA detection.

Using an objective and clinically scalable tool to classify pathogenic alterations in ctDNA, level 1–4 variants opening new potential targeted therapy opportunities were detected in the majority of the patients (57%). In this series, frequencies of each genomic alteration in ctDNA were largely similar to those observed in unselected lung adenocarcinoma populations using tissue-based NGS [8], although with some exceptions. For instance, we did not detect any case with *ALK* or *RET* rearrangements. As there is no significant difference in the detection performance of fusion variants compared with other types of genomic alterations with the Guardant360 assay [6] (we did indeed detect a patient with a *ROS1* fusion), this could be at least partially explained by the fact that our cohort was probably not large enough to represent all the low frequency of NSCLC genomic subgroups. Although comparable, the proportion of patients with actionable alterations is slightly lower than that reported in other series using the same plasma-based NGS assay [10–12]. This might be a result of using more inclusive criteria to define what is actionable (perhaps clinically less realistic), and/or the inclusion of a more clinically selected cohort of patients more likely to harbor actionable variants in these studies.

OncoKB regularly updates the levels of evidence of actionable alterations. For instance, *EGFR* exon 20 insertions and *BRAF* V600E mutations have been recently reassigned to level 3 and level 1 alterations, respectively (formerly level 4 and level 2A, respectively). As the clinical activity of poziotinib had already been

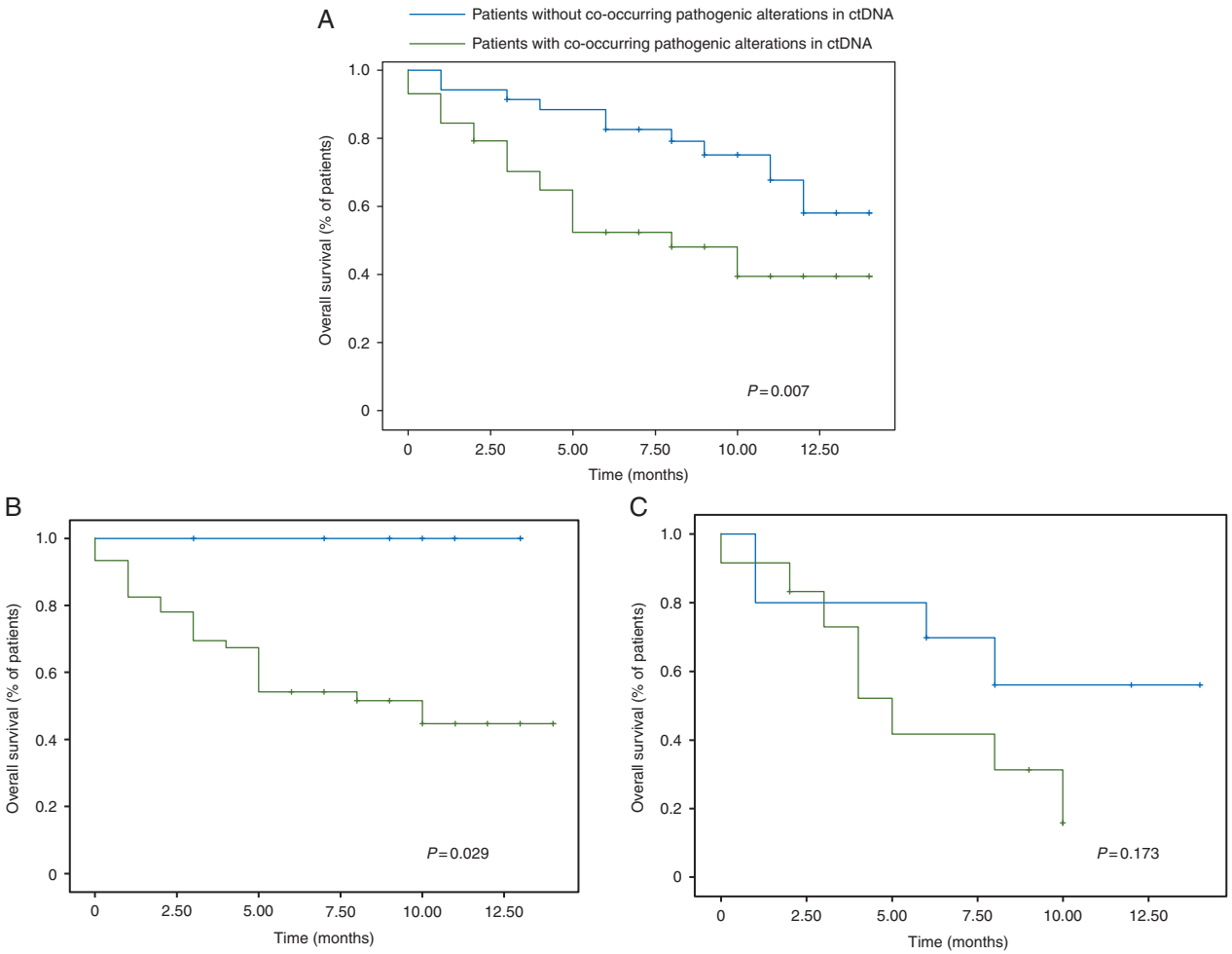

Figure 2. Prognostic performance of the presence of co-occurring pathogenic alterations in (A) entire cohort, (B) subgroup of patients with actionable alterations and (C) subgroup of patients with non-actionable alterations.

reported during the study period [14], we did annotate *EGFR* exon 20 insertions as level 3 alterations. However, in the case of *BRAF* V600E mutations, we categorized them as level 2A alterations based on the available evidence at the time this study was conducted.

Importantly, the specificity and positive predictive value for ctDNA mutation detection of the Guardant360 NGS panel are close to 100% [6], meaning that confirmatory tumor biopsies are not needed to initiate a targeted therapy when an actionable alteration is detected in plasma [5]. Thus, NGS of ctDNA resulted in a therapeutic shift toward genotype-matched treatments in 13% of the patients in the entire cohort (22% of those with actionable alterations). Of note, 12% of the patients that were treatment naive at the time of ctDNA analysis received targeted drugs, underscoring the utility of NGS of ctDNA to select effectively matched first-line targeted therapies. As expected, the use of matched targeted therapies was directly associated with patients' level of actionable variant. The availability of a larger number of genotype-driven trials, and/or an easier access to matched drugs outside the context of a clinical study in our setting, would have probably increased the number patients being treated with targeted drugs. Notably, only three patients (3%) missed the opportunity of potentially receiving targeted therapies due to clinical deterioration during ctDNA NGS. This failure rate is generally higher using tissue-based NGS panels, likely due to substantially longer turnaround times with tissue sequencing [4].

The majority of the patients in this cohort had more than one pathogenic variant in ctDNA (62%). This proportion was remarkably high among the subgroup of patients with actionable alterations (87%), even within those with level 1 variants (90%). These results are consistent with the findings of a large-scale study using ctDNA sequencing (Guardant360) in *EGFR*-mutant NSCLCs, where co-occurring genomic events were detected in up to 93% of the patients [15]. Interestingly, we found no significant difference in the number pathogenic variants between the subset of patients included in level 1 actionable subgroup, a category that includes theoretically more genomically simple tumors, as compared with patients in level 2–4 actionable groups, categories enclosing more genomically complex tumors. These findings further argue against the classic view of oncogene-driven lung adenocarcinomas as single oncogene-driven diseases [15]. Importantly as well, we found that the co-existence of more than one pathogenic alteration was an independent predictor of shorter OS, particularly in the subgroup of patients with actionable alterations. Unfortunately, the number of patients receiving matched targeted drugs and their follow-up period was too low

to assess the relative impact of genomic co-alterations in therapy outcomes, although we did observe that among the subgroup of patients with level 1–2A alterations who received matched targeted therapies, there was a trend to poorer survival in those harboring additional pathogenic co-alterations in ctDNA (supplementary Figure S4, available at *Annals of Oncology* online). Overall, these results add to the growing evidence on the negative influence of co-existing deleterious events in the prognosis and the efficacy of targeted therapies in patients with oncogene-driven lung cancers [15–17].

In conclusion, NGS of ctDNA (Guardant360) is a clinically useful tool to screen for actionable alterations and effectively select lung adenocarcinoma patients for genotype-matched therapies as an alternative to tumor genotyping when tissue is unavailable. Prospective studies addressing the role of genomic co-alterations in predicting outcomes to targeted agents in patients with oncogene-driven cancers are needed.

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