Genotype-specific differences in circulating tumor DNA levels in advanced NSCLC

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Abstract

Introduction—Plasma-based circulating tumor DNA (ctDNA) is an established biomarker for molecular profiling with emerging applications in disease monitoring in multiple tumor types, including non-small cell lung cancer (NSCLC). However, determinants of ctDNA shedding and correlation with tumor burden are incompletely understood, particularly in advanced-stage disease.

Methods—We retrospectively analyzed ctDNA-based and tissue-based genomic data and imaging from 144 patients with NSCLC. Tumor burden was quantified with CT and brain MRI for the overall cohort, and 18F-FDG PET/CT in a subset of patients.

Results—There was a moderate but statistically significant correlation between ctDNA variant allele frequency (VAF) and multiple imaging measures of tumor burden such as CT volume (rho = 0.34, p 0.0001) and metabolic tumor volume (rho = 0.36, p = 0.003). This correlation was strongest in *KRAS* mutant tumors (rho = 0.56, p 0.001), followed by *TP53* mutants (rho = 0.43, p 0.0001), and weakest in *EGFR* mutated (*EGFR*+) tumors (rho = 0.24, p = 0.077). *EGFR*+ tumors with *EGFR* copy number gain (CNG) had significantly higher VAF than *EGFR*+ without CNG (p 0.00001). In multivariable analysis, *TP53* and *EGFR* mutations, visceral metastasis, and tumor burden, were independent predictors of increased ctDNA shedding.

Conclusions—Levels of detectable ctDNA were impacted not only by tumor burden, but also by tumor genotype. The genotype-specific differences observed may be due to variations in DNA shedding and/or cellular turnover. These findings have implications for the emerging use of ctDNA in NSCLC disease monitoring and early detection.

Keywords

lung cancer; ctDNA; liquid biopsy; genotype; PET/CT; tumor volume; cancer detection

INTRODUCTION

Plasma-based circulating tumor DNA (ctDNA) is a highly specific non-invasive biomarker that is increasingly used in multiple solid tumor types for molecular profiling[1, 2] It is also emerging as a promising marker for disease monitoring [3–8] and predicting response to systemic therapy [9–11]. Specifically in non-small cell lung cancer (NSCLC), targeted next-generation sequencing of ctDNA is clinically used as a "liquid biopsy" to identify *EGFR, ALK, ROS1, BRAF, MET, RET, ERBB2*, and other oncogenic targets for therapy in advanced-stage disease [12, 13]. Early changes in ctDNA levels of advanced NSCLC patients treated with immunotherapy and targeted therapy have also been shown to be prognostic [9–11, 14] Additionally, ctDNA-derived tumor mutation burden (TMB) has shown promise as a predictive biomarker for immunotherapy response [15–18]. In early-stage NSCLC, ultra-sensitive ctDNA assays have enabled on-going development of ctDNA as a biomarker by our group and others for detection of molecular residual disease (MRD) after resection [3, 4, 19] and for prediction of pathologic response to neoadjuvant immune checkpoint blockade [10].

Despite these advances, our understanding of critical features relating to ctDNA dynamics, such as determinants of shedding and correlation with tumor burden, remains incomplete. Tumor characteristics that may impact ctDNA levels include size / tumor burden, tumor type, histology, anatomic location, and metabolic activity [1, 20]. The relative contribution of each of these factors and associated characteristics such as genomic subtypes are not well defined. In early-stage lung cancer, plasma ctDNA concentration (as measured by the variant allele frequency, VAF) has been shown to correlate with radiographic tumor volume [3, 4], high Ki67, and presence of lymphovascular invasion [3], using highly customized ctDNA assays. However, there are conflicting data regarding histology as a predictor of ctDNA levels [3, 21]. Furthermore, a quantitative correlation between ctDNA VAF with tumor burden in advanced NSCLC has not been described.

In this study, we sought to comprehensively characterize clinicopathologic, anatomical and functional imaging, and genomic correlates of ctDNA shedding in advanced NSCLC.

MATERIALS AND METHODS

Patients

We reviewed genomic profiling results of advanced NSCLC patients that underwent clinical ctDNA testing at M.D. Anderson Cancer Center between November 2015 and July 2017.

144 consecutive cases with evaluable imaging were selected, prioritizing *EGFR* and *KRAS* mutant cases to ensure sufficient representation for statistical comparisons of these major genomic subtypes. ctDNA testing was obtained generally prior to initiating treatment for newly diagnosed patients or upon disease progression for patients undergoing treatment. Each patient in the study cohort only had a single ctDNA testing time point included in this analysis. This study was conducted in accordance with the institutional review board at The University of Texas M.D. Anderson Cancer Center (PA13-0589 and PA16-0061).

Cell-free circulating tumor DNA assay

ctDNA sequencing was performed with Guardant360 (Guardant Health; Redwood City, CA), a proprietary ctDNA NGS assay that detects single nucleotide variants (SNV) in 70-73 genes as well as select copy number amplifications, indels, and fusions (Supplemental Table S1) [22, 23]. The majority of samples (about 80%) were tested by a 70-gene panel; samples after November 2016 were generally analyzed with the expanded (73-gene) panel. At least 5 ng of cell free DNA was required for hybrid capture-based next generation paired-end sequencing of 160 – 170 base pair DNA strands with average coverage of 8,000x-15,000x depending on the test version. Germline variants were quantitatively excluded, as previously described [22, 23].

Imaging measurements of tumor burden

Tumor burden for each case was quantified using radiographic tumor measurements from CT, 18F-FDG PET-CT, and/or brain MRI scans obtained as part of standard clinical care. Only patients with a CT and/or PET-CT scan within 30 days of ctDNA sample collection were included. RECIST sum of longest diameters (SLD) and tumor volume calculated by semi-automated contouring on CT (syngo.via, Siemens; Malvern, PA) were measured for each case. For cases with available PET imaging, metabolic tumor volume (MTV), maximum SUV (SUV_{max}), and Total Lesion Glycolysis (TLG) were also obtained. MTV and TLG have shown prognostic utility in various malignancies, including NSCLC [24] MTV and TLG were measured by (MIM v.6, MIM Software; Cleveland, OH). TLG was defined as the product of mean SUV and MTV [25]. SLD was defined as the sum of the longest diameters of up to five target lesions (maximum two per organ, including short axis diameters of lymph nodes with short axis ≥ 1.5 cm per RECIST v1.1) [26].

Statistical analysis

For all statistical analyses, ctDNA VAF for each sample was defined as the maximum VAF of the ctDNA alterations detected. All somatic alterations were considered, including variants of unknown significance (VUS) and synonymous mutations. The Wilcoxon rank-sum test and Kruskal-Wallis test were used to compare ctDNA VAF. Spearman's correlation coefficients were determined to assess the relationship between ctDNA VAF and tumor measurements. All tests were two-sided and a p value ≤ 0.05 was considered to be statistically significant. Statistical analyses were conducted using R (version 3.3.2, Vienna, Austria). This study was conducted in accordance with the institutional review board at The University of Texas M.D. Anderson Cancer Center.

RESULTS

Baseline characteristics and summary statistics

The overall study cohort is mostly comprised of previously treated patients (68.1%) that generally had progressive disease at the time of ctDNA testing (Table 1). The prevalence of histologic and major genomic subtypes in the cohort is generally consistent with known prevalence in NSCLC, though, given our study design, *EGFR*-mutated cases are relatively over-represented (40% vs 17% in adenocarcinoma cases of the Pan-Lung Cancer NGS study [27]). For each case, maximum VAF detected in the sample was generally low (median 2.0%, range 0 – 72.5%) and median tumor burden as approximated by RECIST SLD was 6.3 cm (0 – 19.2 cm) and by CT volume was 27.4 cm^3 (0 – 330.5 cm^3).

Of the patients with NCCN guideline recommended biomarkers[28] detected by ctDNA, nine patients (6%) had *MET* amplification, six (4%) had *EGFR* exon 20 insertion, four (3%) had *MET* exon 14 skipping, two (1%) had *HER2* amplification, one (1%) had *HER2* exon 20 insertion, and one (1%) had *NTRK1* fusion (Supplemental Table S4). In the overall study cohort, seven patients (4.9%) had no detectable ctDNA somatic alteration. Eighty patients (55.6%) had prior tissue molecular profiling results available that helped inform their overall mutation status (e.g. *TP53, KRAS*, or *EGFR* mutated). In those cases, the overall mutation status was determined by the union of the ctDNA and tissue somatic mutations.

Radiographic quantifications of tumor burden are associated with ctDNA shedding

We quantified tumor burden using five different approaches: RECIST SLD, CT volume, metabolic tumor volume (MTV), SUV$_{max}$, and Total Lesion Glycolysis (TLG). Within the subgroup of patients that had both CT and PET imaging evaluable (n = 69), each of these radiographic measurements positively correlated with ctDNA VAF, with Spearman's rho ranging from 0.273 (SUVmax) to 0.414 (RECIST SLD); Supplemental Fig. S1 and Fig. 1. When assessing the overall advanced-stage cohort, CT volume had the strongest correlation (Spearman's rho = 0.336, p   0.00001; Fig. 1) and was an independent predictor of ctDNA shedding in a stepwise multivariable regression accounting for visceral metastasis and genomic subtype (Table 2). While the positive correlation between VAF and CT volume was seen across all of the genomic subtypes analyzed, it was strongest in *KRAS* mutant cases (rho = 0.56, p   0.001) and weakest in AGFR-mutated tumors (rho = 0.24, p = 0.077); Supplemental Fig. S2.

Visceral metastasis is associated with increased ctDNA shedding

Patients with visceral metastasis (defined in this study as hepatic, adrenal, renal, or splenic metastasis) had larger tumor burden (as approximated by CT volume, Wilcoxon p   0.001; figure not shown) and increased ctDNA VAF (Wilcoxon rank sum test p   0.001; Fig. 2). Stepwise multivariate Cox regression modeling, accounting for mutation status, and tumor burden, affirmed visceral metastasis as an independent predictor of increased ctDNA shedding (Table 2). Notably, there were numerically higher ctDNA levels observed in patients with the CNS as at least one metastatic site (Wilcoxon p = 0.100; Fig. 2c), though CNS metastasis was not significant in the final regression model. Furthermore, we assessed the impact on ctDNA VAF due to tumor distribution not reflected in RECIST SLD: pleural

effusions (moderate or large) and bilateral sub-centimeter pulmonary nodules (< 10 nodules). There was no statistically significant increase in ctDNA shedding in the presence of those tumor burden characteristics. Additionally, we detected no significant impact on ctDNA shedding due to histology (adenocarcinoma vs squamous cell carcinoma).

Oncogenic mutations in *EGFR* and *TP53* are associated with increased ctDNA shedding

We next compared ctDNA shedding in subgroups defined by the three most common genomic alterations in our cohort: *KRAS, TP53*, and *EGFR.* The *KRAS* and *TP53* subgroups consisted of all patients with non-synonymous somatic mutations in those genes. Only patients with canonical *EGFR* driver mutations (e.g. L858R, exon 19 deletion, G719X, S768I, L861Q, and T790M resistance mutation; no exon 20 insertions) were included in the *EGFR* subgroup. Significantly higher ctDNA VAF was observed in *TP53* mutant vs wild-type tumors (Wilcoxon p 0.001), while numerically higher VAF were observed for the *EGFR*, and *KRAS* subgroups (p = 0.17 and p = 0.16 respectively; Fig. 3a). To verify that these observations were not due to differences in tumor burden, we divided CT tumor volume into tertiles (low, mid, and high volume) and assessed VAF. Median CT volume for the low, mid, and high tertiles were 2.6 cm^3, 28.3 cm^3, and 95.6 cm^3, respectively. For the TP53-mutated and *EGFR*-mutated subgroups, numerically higher VAF was observed across the tumor burden spectrum as approximated by CT volume (Fig. 3b). In the multivariate analysis, TP53-mutated and *EGFR*-mutated status were affirmed as strong, independent predictors of increased ctDNA shedding (Table 2).

Tumors with *EGFR* mutations are known to commonly have copy number gains (CNGs); observed in 28.3% of the *EGFR*+ cases in this cohort. We examined the impact of CNGs on ctDNA levels in *EGFR*+ tumors. EGFR+/CNG+ tumors had a significantly higher VAF than EGFR+/CNG-tumors (Tukey's HSD p 0.00001) (Fig. 4a) . Furthermore, *EGFR*+/CNG-tumors had a VAF comparable to *EGFR* wt/CNG-tumors, suggesting that *EGFR* CNGs were an important contributor to the increased VAF in *EGFR*+ tumors. Furthermore, to determine whether concurrent *TP53* mutations may be driving increased shedding in the *EGFR*+ tumors, we compared *EGFR*+/*TP53* mutant vs *EGFR*+/*TP53* wt tumors. *TP53* co-mutation had no significant additive effect on ctDNA VAF (Fig. 4b).

Patients undergoing active treatment may have decreased ctDNA shedding and thus potentially impact these genotype-specific findings. To further evaluate this potential interaction, we performed additional subgroup analyses with (1) patients that were treatment-naïve and (2) patients that had not received treatment within a month prior to ctDNA testing (treatment-naïve plus "prior treatment" as defined in Table 1). These analyses affirmed differential ctDNA shedding in *TP53* and *EGFR* mutants; though with diminished statistical significance, as expected due to smaller sample size of the subgroups (Supplemental Fig. S3 – S6, Table S7).

DISCUSSION

As clinical uses of ctDNA continue to rapidly mature and incorporate more challenging applications such as MRD detection, disease monitoring, and cancer screening and localization, it is critical to further our understanding of tumor factors that may contribute to

ctDNA shedding heterogeneity. In lung cancer, insight about specific histologic, anatomic, tumor burden, and genomic determinants of ctDNA shedding remains incomplete. In this retrospective analysis, we leverage comprehensive patient-level data of an advanced-stage NSCLC cohort with ctDNA testing results to further our understanding of the relationship between ctDNA detection and key clinicopathological features such as tumor volume and genotype.

A strong correlation between ctDNA VAF and tumor volume has previously been demonstrated in early-stage NSCLC [3, 4]. However, how this correlation holds in advanced-stage disease is not well understood, particularly since volumetrics and other comprehensive measures of metastatic tumor burden are not routinely reported in the clinical setting. We observed that ctDNA VAF positively correlated with metastatic tumor burden as quantified by various radiographic measures including functional assessments of metabolic tumor volume (MTV), SUV, and TLG. To our knowledge, this is the largest analysis of ctDNA correlation with comprehensive radiographic tumor metrics in advanced NSCLC to date. It can be reasonably speculated that non-functional imaging such as CT scans may not be able to accurately measure systemic tumor burden, since observed radiographic lesions may be comprised of necrotic cells or non-neoplastic cells (e.g. stromal, inflammatory) [29] It is therefore notable that tumor burden approximated by anatomical CT volumetrics was comparable to PET-based volumetrics in ctDNA fraction correlation. Furthermore, the ubiquitous RECIST SLD measurement also appeared to comparably approximate overall tumor burden, as correlated by ctDNA fraction.

While positive, the modest correlation observed between VAF and tumor burden in our cohort suggests that other factors also significantly impact ctDNA shedding. Our findings that visceral metastasis predicts for increased ctDNA shedding is consistent with prior studies correlating liver metastasis with increased ctDNA levels in NSCLC [30, 31]. While vascular accessibility may facilitate tumor ctDNA release, poor tumor vascularization may also contribute to ctDNA shedding in the setting of hypoxia and cell death.

Additionally, liver metastasis, in particular, may possibly be associated with high ctDNA VAF because of attenuation in the hepatic clearance of ctDNA, since the liver is the primary mechanism of ctDNA removal [32] In contrast, histology, brain metastasis, and significant pleural effusions or bilateral sub-centimeter pulmonary nodules did not appear to independently impact ctDNA levels in our study.

We observed genotype-specific differences in ctDNA levels in our cohort, potentially highlighting underlying differences in tumor biology mediated by metabolic activity and DNA copy number alterations. The finding that *TP53* mutations independently predict for increased ctDNA shedding, even after accounting for tumor burden and visceral metastasis, may reflect the increased cellular turnover or metabolic activity of these tumors. *TP53* mutation (or overexpression) has been considered a marker of poor prognosis but this assessment has been hampered by an evolving understanding of how best to accurately evaluate *TP53* function and status [33, 34] Moreover, our observations about increased ctDNA shedding in *EGFR* mutated tumors and possible interactions due to concomitant *EGFR* copy number gains have implications for the use of ctDNA as a biomarker in this important

genomic subgroup. Focal amplifications of specific chromosomal regions, such as *EGFR* CNGs, result in an increased fraction of detectable ctDNA in plasma, thus potentially over-estimating tumor volume and biasing ctDNA-derived measures of clonality [35] and tumor mutation burden, a biomarker with predictive utility across multiple tumor types [15–18, 36]

Our findings of differential shedding also have ramifications for one of the major emerging clinical applications of ctDNA: cancer detection. Our group and others have shown that ultra-sensitive ctDNA assays can effectively detect MRD after curative intent therapy in localized NSCLC, thus identifying patients with highest risk for subsequent relapse [3, 4, 19] Other ctDNA-based platforms further optimize DNA fragment size and methylation analyses to enable detection of minimal levels of ctDNA and thus potential population-scale cancer screening [37, 38]. Clinical validation of these emerging ctDNA applications will need to evaluate and potentially stratify for heterogeneity in the various determinants of shedding in order to accurately demonstrate clinical utility. For example, early detection of recurrence may be missed in patients presenting primarily with pleural effusions or sub-centimeter pulmonary nodules. Conversely, overrepresentation in the number of patients presenting with visceral metastasis or tumors with CNGs may exaggerate the ability of a particular assay to detect small tumor volumes. Additionally, since clinical trials required to prospectively validate cancer screening tests are enormously time and resource intensive [39, 40], *in-silico* modeling and simulation are useful for projecting ctDNA test performance characteristics such as minimal tumor detection volumes [41]. Observations about determinants of ctDNA shedding are critical to inform these models.

Our study has several possible limitations. Since our cohort is mostly comprised of previously treated patients (68%), generally with progressive disease at the time of ctDNA testing, there may be a higher prevalence of resistance alterations (e.g. *MET* amplifications) that may impact ctDNA levels but not accounted for in the current analysis. Additionally, our KRAS-mutated cohort reflects the known heterogeneity of *KRAS* in lung adenocarcinoma. We have previously shown that *KRAS* subgroups (characterized by inactivation of *TP53, STK11, KEAP1*, and *CDKN2A/B)* indeed have distinct cancer biology [42]. A larger *KRAS* cohort may better delineate potential ctDNA shedding differences between these *KRAS* subgroups. Finally, while the clinical ctDNA assay used in our study bioinformatically excludes variants associated with clonal hematopoiesis (CH), the potential inclusion of these non-tumor-related somatic variants in our analyses cannot be definitively quantified since matching white blood cells were not available for further DNA sequencing.

Despite these limitations, our findings strongly suggest that ctDNA shedding is not only impacted by tumor burden but also by anatomic location and genomic subtype. These findings further the understanding of ctDNA biology in NSCLC and have implications for the rapidly emerging use of ctDNA in NSCLC MRD, disease monitoring, and early detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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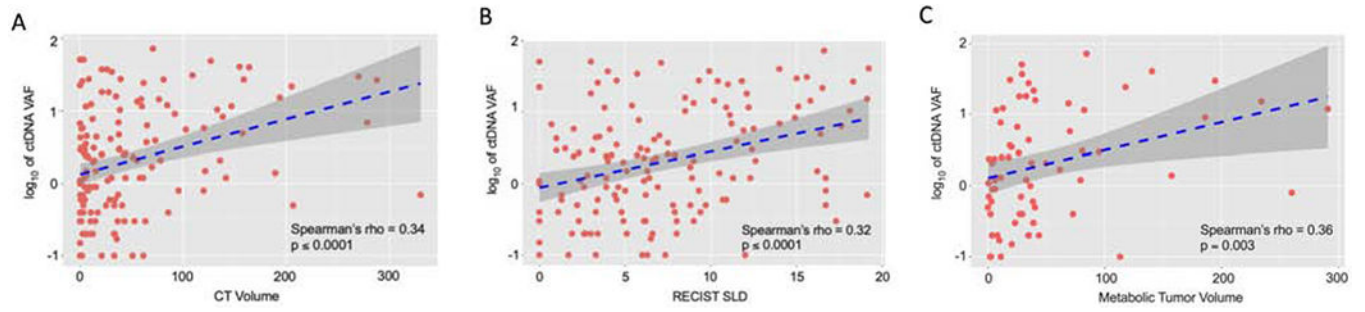
Figure 1.
Correlation between ctDNA VAF and tumor burden as approximated by **(A)** CT volume (cm^3) **(B)** RECIST SLD (cm) in the overall cohort and **(C)** MTV (cm^3) in the PET subgroup; Spearman's rho correlation.
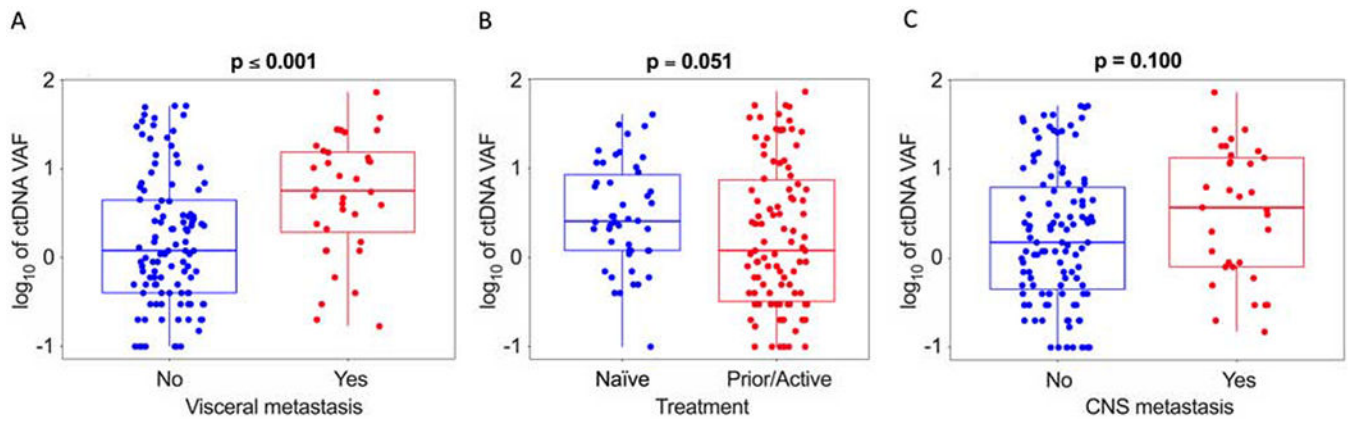
Figure 2.
Impact of (**A**) visceral metastasis, (**B**) treatment status, and (**C**) CNS metastasis on ctDNA VAF. Wilcoxon rank-sum test.
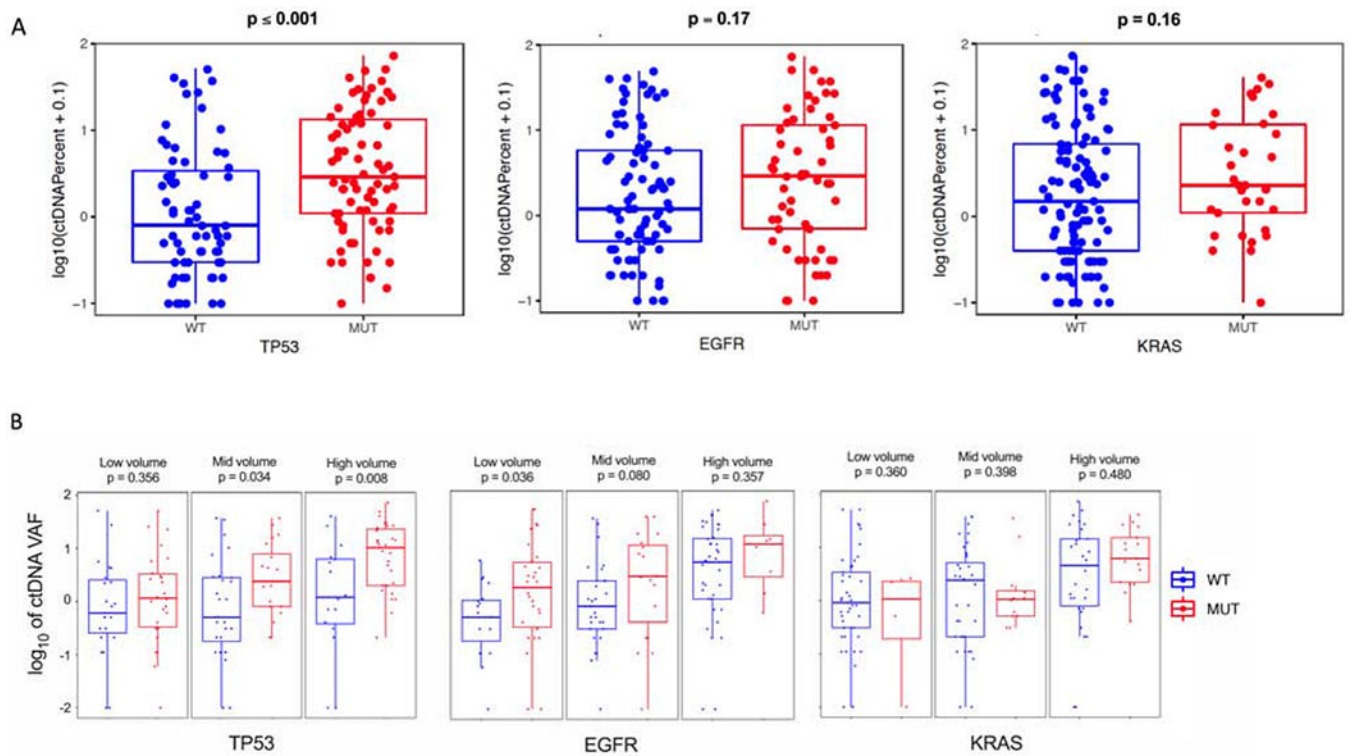
Figure 3.
ctDNA VAF comparison across genomic subtypes. **(A)** Wilcoxon rank-sum test for *TP53, EGFR*, and *KRAS* mutated tumors and **(B)** Wilcoxon rank-sum test in *TP53, EGFR, and KRAS* mutated cases by CT volume (low [0-10 cm^3], mid [10-50 cm^3], high [> 50 cm^3]).
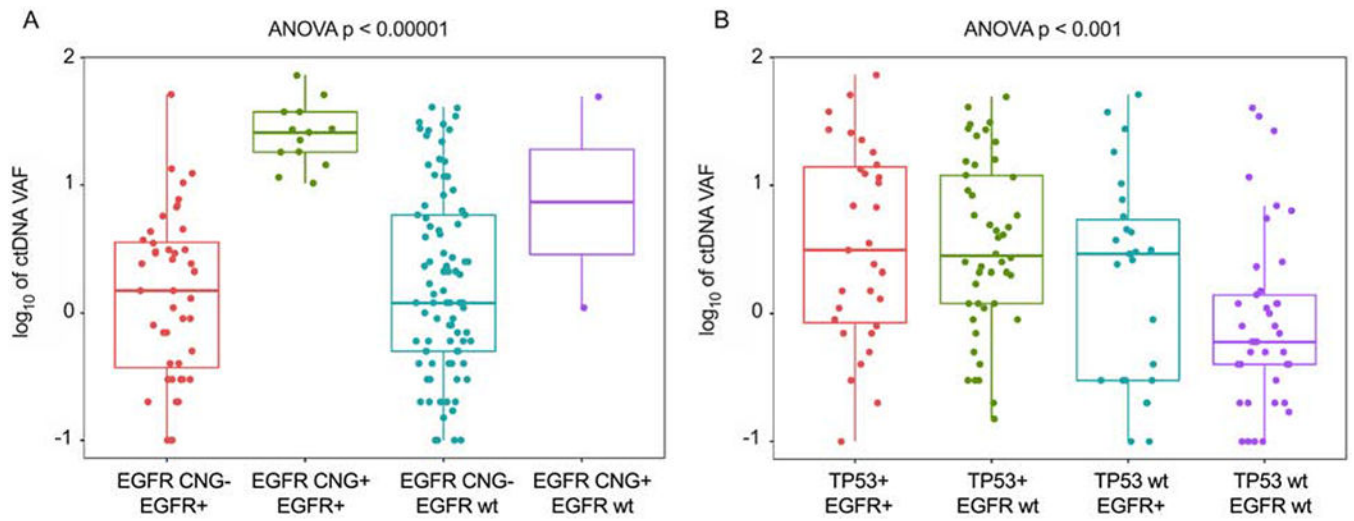
Figure 4.
Impact of co-alterations on *EGFR*-mutated shedding. **(A)** Impact of *EGFR* copy number gain and **(B)** *TP53* co-mutation on *EGFR*-mutated shedding; ANOVA comparison.

Table 1:

Baseline Characteristics of Overall Study Cohort

Characteristic (n = 144)	Number (%)
Median age (range)	64 (28 – 96)
Sex	
Female	78 (54.2)
Male	66 (45.8)
Histology	
Adenocarcinoma	112 (77.8)
Squamous cell	31 (21.5)
Other NSCLC	1 (0.7)
Alteration [a]	
TP53 mutation	77 (53.5)
KRAS mutation	33 (22.9)
EGFR mutation	57 (39.6)
ALK fusion	4 (2.8)
Treatment status	
Treatment naïve	46 (31.9)
Active treatment [b]	77 (53.5)
Prior treatment [c]	21 (14.6)
Immunotherapy exposure	
No	118 (81.9)
Yes	26 (18.1)

[a] Sum is greater than number of patients due to co-mutations

[b] Systemic treatment within 1 month prior to ctDNA testing

[c] Systemic treatment more than 1 month prior to ctDNA testing but within last 2 years

Table 2:

Univariate and multivariate analysis of ctDNA VAF

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR 95% CI	P-value
Histology	0.94 (0.70 – 1.27)	0.694		
Immunotherapy exposure	1.03 (0.75 – 1.43)	0.845		
Visceral metastasis	1.66 (1.27 – 2.19)	< **0.001**	1.48 (1.16 – 1.91)	**0.002**
CNS metastasis	1.33 (1.00 – 1.79)	0.053		
RECIST SLD	1.05 (1.03 – 1.08)	< **0.0001**		
CT volume	1.00 (1.00 – 1.00)	< **0.0001**	1.00 (1.00 – 1.00)	< **0.001**
TP53+	1.58 (1.25 – 2.00)	< **0.001**	1.45 (1.16 – 1.80)	**0.001**
EGFR+	1.19 (0.92 – 1.53)	0.179	1.36 (1.08 – 1.71)	**0.009**
KRAS+	1.22 (0.91 – 1.64)	0.178		