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Associations between tumor mutations in cfDNA and survival in non-small cell lung cancer☆

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ABSTRACT

Introduction: Studies have indicated that detection of mutated *KRAS* or *EGFR* in circulating tumor DNA (ctDNA) from pre-treatment plasma samples is a negative prognostic factor for non-small cell lung cancer (NSCLC) patients. This study aims to investigate whether this is the case also for NSCLC patients with other tumor mutations.
Methods: Tumor tissue DNA from 107 NSCLC patients was sequenced and corresponding pre-treatment plasma samples were analyzed using a limited target next-generation sequencing approach validated in this study. Patients without detected mutations in tumor samples were excluded from further analyses.
Results: Mutations were detected in tumor samples from 71 patients. Median age was 68 years, 51% were female, and 88% were current/former smokers, 91% had adenocarcinoma, 4% had squamous cell carcinoma and 6% had other NSCLC. The distribution between stage I, II, III and IV was 33%, 8%, 30%, and 29%, respectively. Between one and three tumor mutation(s) were detected in ctDNA from corresponding plasma samples. Patients with detected ctDNA had shorter PFS (9.6 vs. 41.3 months, HR: 2.9, 95% CI: 1.6–5.2, $p = 0.0003$) and OS (13.6 vs. 115.0 months, HR: 4.0, 95% CI: 2.1–7.6, $p = 0.00002$) than patients without detected ctDNA. ctDNA remained a significant negative prognostic factor for OS (HR: 2.5, 95% CI: 1.1-5.7, p=0.0327), but not PFS, in the multivariable analyses adjusting for baseline patient and disease characteristics including stage of disease.
Conclusions: This study adds further evidence supporting that detectable tumor mutations in cfDNA is associated with a worse prognosis in NSCLC harboring a variety of tumor mutations.

Introduction

The treatment for patients with non-small cell lung cancer (NSCLC) is mainly recommended based on assessment of TNM stage of disease, molecular markers, WHO performance status, and comorbidities [1, 2]. Even if the TNM staging system is based on growing databases and has become more detailed in recent years, patients with the same TNM stage receiving similar treatment still have different outcomes [3].

Circulating tumor DNA (ctDNA) originates in tumor cells and leaks into the circulation [4]. Studies indicate that detection of ctDNA by identification of tumor mutations in plasma collected before treatment is a negative prognostic factor for patients with lung adenocarcinoma [5–7]. Most studies, including one by our group [8], have investigated NSCLC patients with mutations in *KRAS* or *EGFR*. Notably, most NSCLC patients harbor other mutations than *KRAS* or *EGFR*, and the mutation spectrum is heterogeneous between patients [9,10]. ctDNA accounts for a minority of total cell-free DNA (cfDNA) found in plasma and requires sensitive methods for its detection [11]. Droplet digital polymerase chain reaction (ddPCR) is a highly sensitive method for detecting mutations in cfDNA, but it is laborious to analyze samples with different genes. Next-generation sequencing (NGS) allows analysis of any number of genes in each sample and enables analysis of different genes in several samples simultaneously. However, NGS is rather expensive since the detection of low-frequency mutations requires high genome coverage. The cost also depends on the number of target regions, i.e., the number of genes or mutations screened. To keep the costs at an acceptable level,

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we tested and validated an approach to limit the number of target regions in cfDNA to the region(s) found to contain a mutation in the patients' tumor tissue DNA.

The main aim of this study was to investigate whether detectable ctDNA in a pretreatment plasma sample was associated with progression-free survival (PFS) and overall survival (OS) in NSCLC patients with a minimum of one mutation in their tumor DNA using this targeted NGS approach.

Methods

Patients and approval

This study was approved by the Regional Committee for Medical and Health Research Ethics (REC) in Central Norway. Patients were included in our regional research biobank, Biobank1, approved by the REC in Central Norway, the Ministry of Health and Care Services, and the Norwegian Data Protection Authority. Participants are over 18 years old and have given written informed consent.

One hundred and seven patients registered as having adenocarcinoma, diagnosed between March 2007 and April 2018, were included. These were the patients registered with adenocarcinomas in our biobank from which tumor tissue and pretreatment plasma samples were available for analyses. Patients with a tumor mutation in *KRAS* codon 12/13 included in another study were excluded from this study [10]. We considered this sample size and follow-up time sufficient for this exploratory study. All tumor specimens were reviewed and classified according to the WHO 2015 classification system. The disease stage was assessed according to the Eighth Edition of the TNM Classification of lung cancer [14].

Test and validation of limited target next-generation sequencing

We used NGS to analyze mutations in plasma cfDNA that were previously found in the patient's tumor DNA. A limited number of target regions in cfDNA covering the positions of the identified tumor mutation (s) were sequenced by selecting a set of region-specific primers for each patient. Before analyzing patient cfDNA samples, we tested if this approach was feasible. The sensitivity of the NGS approach was investigated by sequencing constructed DNA solutions with different mutant allele frequencies of a *KRAS* mutation. ddPCR was used to validate NGS results from the constructed DNA solutions and nine cfDNA samples from *KRAS* mutated NSCLC patients.

We obtained a *KRAS* G12C positive formalin-fixed paraffin-embedded (FFPE) tumor sample with a known mutant allele frequency (MAF) of 9%. We composed four *KRAS* G12C DNA solutions with defined MAF of 1.15%, 0.17%, 0.016%, and 0.0016%, respectively, by diluting the tumor DNA with peripheral blood DNA from the same patient. In this way, we artificially constructed DNA solutions imitating cfDNA samples with known concentrations of ctDNA.

NGS libraries were made using 40–48 ng DNA and reagents from QIAseq Human Targeted DNA panel (Qiagen, Hilden, Germany) except the primer mix. In brief, DNA was fragmented, end-repaired, and a-tailed followed by ligation to a 5′ adapter. Adapters contained a unique molecular index (UMI) that provided a unique tag for all original DNA fragments. The ligated fragments were purified, and the region of interest was selected by PCR using an adapter primer and a 1 nM solution of region-specific primers with a 5′ universal sequence. The concentration of gene-specific primers highly exceeded the concentration of input genomic DNA in the hybridization reaction, ensuring that complementary DNA was formed from all available genomic templates. The PCR products were then purified and amplified in a second PCR using an adapter primer and a primer complementary to the universal sequence. Libraries were sequenced using the MiSeq platform (Illumina, San Diego, CA).

NGS mutation analysis was performed using CLC Biomedical Workbench v.20.0 (Qiagen, Hilden, Germany) with a ready-to-use workflow for QIAseq Targeted DNA panels. Following mapping reads to the genome, any reads outside the region of interest were excluded. Two or more reads with the same UMI were grouped into a "UMI family." Single reads with no duplicates were discarded. Variants were called if 75% of duplicates in a UMI family contained the variant. Only the position of the mutation of interest was considered. A solution was classified as "mutation detected" if *KRAS* G12C was detected in at least one big UMI family, defined as a family made from $\geq$ 4 duplicates. ddPCR (Bio-Rad Laboratories, Hercules, CA) was performed using 40–48 ng constructed cfDNA. NGS was carried out in duplicates and these duplicate solutions were analyzed by ddPCR in quadruplicates. Patient cfDNA was analyzed in triplicates by ddPCR.

Tumor DNa sequencing and region selection

Tumor DNA was isolated from diagnostic FFPE tumor tissue using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) and sequenced using QIAseq Human Actionable Solid Tumor panel (Qiagen, Hilden, Germany). This panel covered the whole coding region of the genes *ERBB2, PIK3CA,* and *TP53*, the exons of *BRAF, EGFR, KIT, KRAS, NRAS,* and *PDGFRA*, and hotspots in *AKT1, ALK, CTNNB1, ERBB3, ESR1, FOXL2, GNA11, GNAQ, IDH1, IDH2, MET, RAF1*, and *RET*. Theoretically, this panel enables the detection of at least one mutation in about 65% of adenocarcinoma tumors and more than 80% of squamous cell carcinoma tumors [9,10].

Bioinformatic analysis was performed using CLC Genomic Workbench 20.0 (Qiagen, Hilden, Germany) with a ready-to-use workflow for QIAseq Targeted DNA panels. Variants were classified as a mutation if the variant was non-synonymous with a MAF of at least 5%. Patients without detected tumor mutation were excluded from further analyses.

For each tumor mutation, we identified the primers used to amplify the region with the mutation. A 5′ universal sequence was added to all primers, and the primers were synthesized by Eurogentec (Liège, Belgium).

Patient cfDNA extraction

Plasma samples from the 71 patients with at least one detected tumor tissue mutation were analyzed. The median time from blood draw to tissue biopsy was one day (range 0–213). At blood draw, plasma was prepared from 10 mL whole blood with EDTA or citrate anticoagulant. Within two hours of sampling, the blood samples were either centrifuged once at 2500x g for 10 min or first at 1500x g for 15 min, and then at 10,000x g for 10 min. Plasma was transferred to cryotubes and stored at -80 °C. cfDNA was isolated using QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) from 1.6 to 6 mL plasma and eluted in 50 uL of the supplied buffer. DNA concentration was measured by Qubit® (Thermo Fisher Scientific, Waltham, MA), using the dsDNA HS Assay Kit.

cfDNA analyses by limited target NGS

NGS libraries were made using a variable amount of cfDNA (5.3–72.5 ng) and as described for the constructed samples. In 63 samples, primers were added to target one region (i.e., one mutation). Two regions were targeted in eight samples and three regions were targeted in three samples. The goal was to achieve the same genomic coverage at each target region in all samples. Therefore, when libraries were pooled before sequencing, we doubled the library amount from the eight samples with two targets. Similarly, we tripled the amount of the libraries with three targets. The number of libraries in each pool was adjusted to generate 700–800,000 reads per target using the MiSeq v3 platform (Illumina, San Diego, CA). NGS data were analyzed as described for the constructed DNA solutions.

Statistics

PFS was defined as the number of days from lung cancer diagnosis until progression or death of any cause. OS was defined as the number of days from diagnosis until death of any cause. Patients were treated and followed according to local routines. The median follow-up time for PFS and OS were estimated using the reverse Kaplan–Meier method, and the median PFS and OS were estimated using the Kaplan-Meier method. Survival analyses were performed using Cox proportional hazard models. The multivariable model was adjusted for sex, age, WHO performance status (PS), and disease stage. The threshold for statistical significance was set at 0.05. All statistical analyses were performed using R (version 3.6.1).

Results

Patient characteristics

A summary of patient characteristics is presented in Table 1. The median age was 68 years (range 48–86), 54 (51%) were female, and 94 (88%) were current or former smokers, 97 patients (91%) had adenocarcinoma, four (4%) had squamous cell carcinoma, one (1%) had adenosquamous carcinoma, and five (5%) had NSCLC not otherwise specified. Thirty-five patients (33%) had stage I disease, nine (8%) stage II, 32 (30%) stage III, and 31 (29%) stage IV. Individual patient characteristics are presented in Table A1.

Validation of limited target NGS

The strategy for ctDNA detection was to use NGS to only analyze for mutations found in the patients' tumor DNA. The performance of this limited target NGS approach was explored by constructing four solutions made to mimic cfDNA with theoretical MAF of *KRAS* G12C at 1.15%, 0.17%, 0.016%, and 0.0016% that were analyzed by both NGS and ddPCR. Comparable results were obtained in the solutions with MAFs 1.15% and 0.17% (Fig. 1A). In addition, the mutation in the solution with MAF 0.0016% was detected using ddPCR. While the input DNA amount was similar in both NGS and ddPCR, the number of unique human genome equivalents (hGEs) analyzed by NGS was 5600–8070 compared to 10,580–17,774 by ddPCR. The partitioning of DNA in ddPCR likely contributed to superior sensitivity.

We proceeded to analyze cfDNA from nine patients with *KRAS*-mutated tumors using both NGS and ddPCR, and comparable results were obtained by the two techniques. An excellent correlation was observed between the observed MAFs by NGS and ddPCR in the constructed solutions and patient samples together (adjusted $R^2 = 0.9944$, $p = 2.2 \times 10^{-16}$) (Fig. 1B). Concordant results between NGS and ddPCR were observed in all samples except one, where a mutation was detected with MAF 0.17% by NGS but not detected by ddPCR. This may be attributed to the different strategies for target amplification. In NGS, amplification required only one target-specific primer to bind to DNA while ddPCR required two. Consequently, short DNA fragments that did not contain both primer sites would not be amplified and analyzed.

Although we did not validate the efficiency for the primers of non-KRAS genes for the NGS analysis of cfDNA specifically, we reasoned from NGS data of tumor DNA that all available genomic templates in cfDNA were converted to complementary DNA when an excess of gene-specific primers were used in the first step of the hybridization reactions. The primers used in the following PCR reactions were the same for the KRAS and non-KRAS genes which ensured similar sensitivity of mutation detection for all genes.

Table 1
Patient characteristics.

	All patients ($n = 107$) Number (%)	Patients included for cfDNA analyses ($n = 71$) Number (%)	Patients with detected ctDNA ($n = 29$) Number (%)	Patients without detected ctDNA ($n = 42$) Number (%)
Age (median, range)	68 (48–86)	68 (50–86)	66 (50–81)	69 (54–86)
Sex				
Female	54 (50)	38 (54)	16 (55)	22 (52)
Male	53 (50)	33 (46)	13 (45)	20 (48)
Smoking history				
Never-smoker	13 (12)	10 (14)	3 (10)	7 (17)
Current or former smoker	94 (88)	61 (86)	26 (90)	35 (83)
Histology				
Adenocarcinoma	97 (91)	65 (92)	26 (90)	39 (93)
Squamous cell carcinoma	4 (4)	3 (4)	2 (7)	1 (2)
Adenosquamous carcinoma	1 (1)	1 (1)	0	1 (2)
NSCLC—NOS	5 (5)	2 (3)	1 (3)	1 (2)
PS				
0	59 (55)	41 (58)	13 (45)	28 (67)
1	40 (37)	26 (37)	12 (41)	14 (33)
2	8 (8)	4 (6)	4 (14)	0
Disease stage				
I	35 (33)	24 (34)	1 (3)	23 (55)
II	9 (8)	7 (10)	2 (7)	5 (12)
III	32 (30)	20 (28)	15 (52)	5 (12)
IV	31 (29)	20 (28)	11 (38)	9 (21)

NSCLC—NOS: non-small cell lung cancer – not otherwise specified, PS: WHO performance status.

Tumor DNA mutation detection and region selection

At least one tumor mutation was detected in 71 patients (66%). One mutation was detected in 60 patients, two mutations in nine patients, and three mutations in two patients. The 85 mutations were detected in the genes *TP53* (54%), *EGFR* (14%), *KRAS* (12%), *PIK3CA* (9%), *BRAF* (4%), *ERBB2* (1%), *ALK* (1%), *ERBB3* (1%), *NRAS* (1%), *PDGFRA* (1%) and *RAF1* (1%). The specific tumor mutations are listed in Table A.2. For each tumor mutation, we identified the panel primers flanking the mutated region. Between one and nine target-specific primers were selected for each patient.

Detection of ctDNA by limited target NGS

NGS libraries were made using 1620–21,982 hGEs (median 6482) and target-specific primers determined by the tumor DNA sequencing. One mutation (i.e., one region) was targeted in cfDNA from 61 patients, two mutations in seven patients, and three mutations in three patients. (Accidently, only one region was targeted in the plasma sample from patient 45 with two detected mutations in the tumor).

The number of UMI families that covered the position of the tumor mutation ranged from 266 to 5955 (median 973). One UMI family represents one hGE from the original sample. On average, the number of UMI families was 22% of the number of hGE used for library preparation (range 2−71%). The loss of hGEs was greater than the loss observed in the constructed solutions (50%). cfDNA is more fragmented and consists of shorter fragments than DNA from whole blood that was used to construct the artificial cfDNA. Therefore, fewer DNA molecules may have been available for the first PCR with gene-specific primer binding in the patient cfDNA samples which contributes to a greater loss.

At least one tumor mutation first detected in tumor DNA was also detected in corresponding cfDNA samples from 29/71 patients (41%). In total, 32 such mutations were detected with MAFs between 0.05% and 65.7% (median 1.8%). The mutation with MAF 65.7% was an exon 20 insertion in *EGFR* in a sample where another mutation in *TP53* with MAF 6.3% was detected. We believe that the high MAF was most likely caused by an an amplification of the *EGFR* gene in the tumor, since this

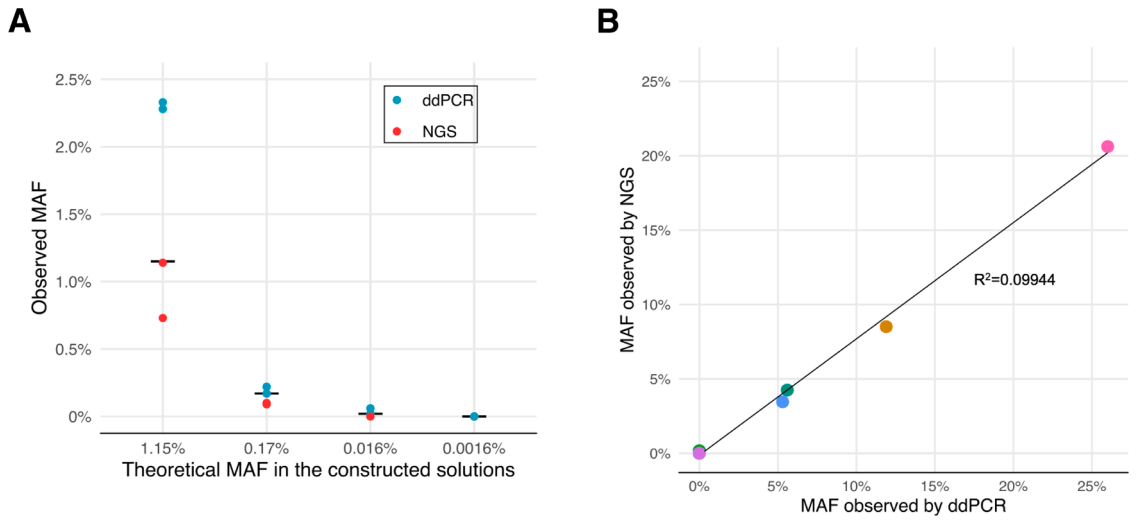

Fig. 1. To test the limited target NGS method, we constructed cfDNA solutions and patient cfDNA samples that were analyzed by both NGS and ddPCR. **(A)** Four DNA solutions were constructed to mimic cfDNA with KRAS G12C mutation with MAF 1.15%, 0.17%, 0.016%, and 0.0016%. Each solution was analyzed in duplicates. The horizontal line represents the theoretical MAF. **(B)** MAF of KRAS codon 12 mutations in nine patient cfDNA samples analyzed by both NGS and ddPCR. The mutation was detected in five samples and undetected by both technologies in four samples. cfDNA: circulating cell-free DNA, ddPCR: droplet digital polymerase chain reaction, NGS: next-generation sequencing, MAF: mutant allele frequency.

mutation has consistently been reported to be a tumor-associated mutation in NSCLC and is often amplified (although a germline mutation cannot be ruled out since we did not sequence normal DNA from our patients). For the other samples, the MAFs ranged from 0.05% to 24.5%. Individual mutation data are listed in Table A.2.

In 8/10 cfDNA samples where more than one region was targeted, either all or none of the tumor mutations were detected. In 2/10 patients (patients 21 and 22), only one mutation was detected in each sample. ctDNA was detected in 1/24 (4%) patients with stage I, 2/7 (29%) with stage II, 15/20 (75%) with stage III, and 11/20 (55%) with stage IV disease.

Association between ctDNA detection and progression-free survival (PFS)

Median follow-up time for PFS was 88.7 months (95% CI: 45.2–105.9) and 23 patients were alive and relapse-free at the time of data analysis in November 2020. Overall, the median PFS was 17.5 months (95% CI: 7.6–126.2). Median PFS was significantly shorter for patients with detected ctDNA than for those without detected ctDNA (9.6 months vs. 41.3 months, HR: 2.9, 95% CI: 1.6–5.2, $p = 0.000325$) (Fig. 2A). In the multivariable analysis, PS and disease stage, but not detectable ctDNA, were significantly associated with PFS (Table 2). In terms of two-year PFS, 83% of patients with detected ctDNA relapsed or died within two years, compared to 38% of patients without detected ctDNA.

Association between ctDNA detection and overall survival (OS)

Median follow-up time for OS was 65.6 months (95% CI 45.2–106.0) and 28 patients were alive at the time of data analysis. Overall, the median OS was 27.5 months (95% CI: 13.0–126.2). Median OS was significantly shorter for patients with detected ctDNA than for patients without (13.6 months vs. 115.0 months (HR: 4.0, 95% CI: 2.1–7.6, $p = 0.0000201$) (Fig. 2B). The multivariable analysis showed that detected ctDNA, stage IV and PS 2 were significant, negative prognostic factors (Table 2).

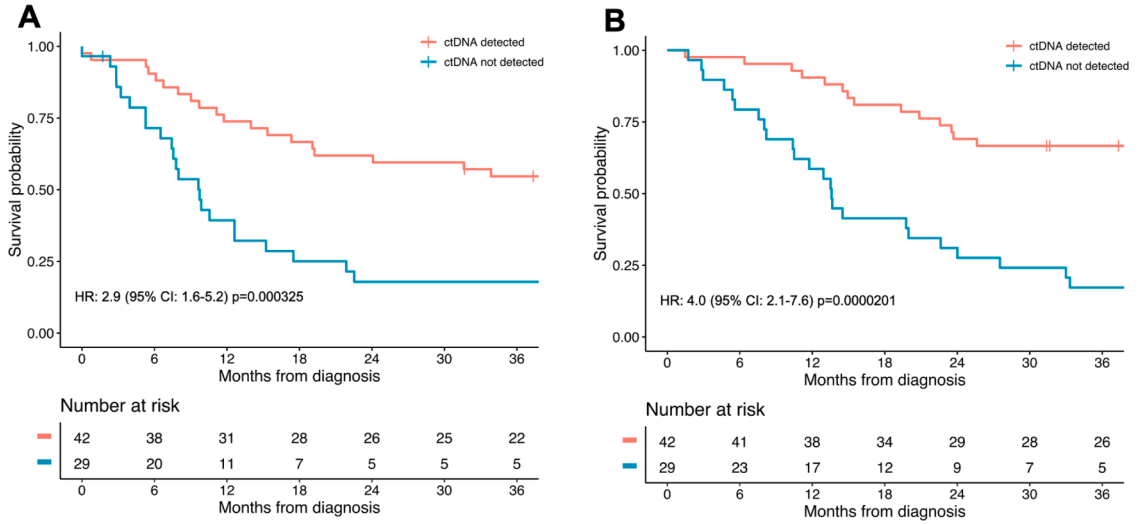

Fig. 2. Kaplan-Meier plot for **(A)** progression-free survival and **(B)** overall survival. CI: confidence interval, ctDNA: circulating tumor DNA, HR: hazard ratio.

Table 2
Cox multivariable model for PFS and OS. Statistically significant values are given in bold.

	Progression-free survival		Overall survival	
	Hazard-ratio (95% CI)	*p*-value	Hazard-ratio (95% CI)	*p*-value
Sex (male vs female)	0.94 (0.52–1.70)	0.827	0.91 (0.48–1.74)	0.785
Age	1.02 (0.98–1.06)	0.227	1.03 (0.99–1.07)	0.160
Stage II vs I	1.07 (0.28–4.13)	0.920	1.16 (0.21–6.33)	0.865
Stage III vs I	2.68 (0.97–7.35)	0.0562	3.13 (0.90–10.91)	0.0727
Stage IV vs I	7.00 (2.81–17.40)	**0.0000286**	9.56 (3.19–28.67)	**0.0000555**
PS (2 vs 1/0)	13.07 (2.59–66.00)	**0.00185**	10.31 (2.09–50.92)	**0.00421**
Detection of ctDNA	1.58 (0.75–3.32)	0.227	2.49 (1.08–5.74)	**0.0327**

CI: confidence interval, ctDNA: circulating tumor DNA, PS: WHO performance status.

Discussion

In this study of 107 patients with all stages of NSCLC, we detected tumor mutation(s) in 66% of the samples, and when sequencing corresponding pre-treatment plasma samples from these 71 patients, we detected the same mutation(s) in 41% of patients. We found that detection of ctDNA in plasma was significantly associated with shorter PFS and OS in the univariable analyses, and ctDNA remained a significant negative prognostic factor for OS in the multivariable analyses.

There are several other studies of the prognostic role of ctDNA in NSCLC. Pavan et al. found that TP53 mutations in plasma detected by NGS, the most commonly detected mutation in ctDNA in our cohort, negatively affected survival both in NSCLC patients who received immune checkpoint inhibitor therapy and those who did not [13]. Michaelidou et al. used ddPCR to analyze *KRAS* mutations in cfDNA from 114 advanced NSCLC patients with tumor *KRAS* status either mutated, wild-type, or unknown, and found detection of mutated *KRAS* in ctDNA to be significantly associated with both PFS and OS [5]. In a previous study by our group, we used ddPCR to analyze matching tumor and plasma samples from 60 patients with known *KRAS* mutations and found that detectable *KRAS* in cfDNA was significantly associated with both PFS and OS [8]. Peng et al. found similar associations by sequencing both tumor tissue and cfDNA from 77 patients with resectable NSCLC using a 127-gene panel [6]. Mardinian et al. used NGS to analyze *KRAS* mutations both in tumor tissue and cfDNA from 433 patients with various cancer types, including NSCLC, and found a significant association between ctDNA detection and shorter OS [7]. Interestingly, they also showed that the value of ctDNA as a prognostic marker was greater when *KRAS* mutation was detected in both tumor tissue and cfDNA, compared to either sample type alone.

However, results are not uniform across all studies. In a study of 58 *KRAS*-mutated NSCLC patients, detection of *KRAS* in cfDNA was not associated with shorter PFS [12], while two recent studies found similar associations as we report here [5,6]. These studies also observed an independent prognostic association between ctDNA detection and PFS. Differences in patient selection with respect to histology and disease stage, and frequency of computed tomography evaluation might explain why results differ.

The main limitation of all these studies, including ours, is the sample sizes, and in particular the number of patients with low disease stage and detectable ctDNA was too low to draw firm conclusions. Another limitation is that we only detected tumor mutations in 71 of the 107 patients included in our study. A broader NGS panel or sampling tissue from different parts of the tumors might increase the mutation detection rate, but the latter is limited by the access to routinely obtained tissue. Analyzing a panel of genes in cfDNA alone could overcome the above-mentioned challenges associated with tumor tissue analysis. On the other hand, limiting the cfDNA analysis to known tumor mutations reduces the chance of detecting false positives in cfDNA. The NGS approach applied in this study was a cost-efficient method for analyses of tumor mutations in cfDNA. In the study by Peng et al. using a 127-gene panel, 1.2 mutations were on average detected in the tumor samples. cfDNA was analyzed for the matching mutations using the same 127-gene panel, which demonstrates that large panel sequencing of cfDNA generates a myriad of uninformative data [6].

Another potential limitation is that we did not assess total tumor volume, which has been shown to be associated with presence of ctDNA [14]. Thus, we cannot rule out that presence of ctDNA is a surrogate marker for large total tumor volume in our cohort. On the other hand, the impact of tumor volume varies with TNM stage and treatment, and tumor volume is not routinely assessed in the clinic.

This was a retrospective study and patients were included in the biobank over a long time period, and there was a large variation in time from plasma samples were collected until biopsies were obtained. There were major changes in diagnostic workup during this period (e.g. PET CT for staging of disease and reflex-testing for EGFR-mutations and ALK-rearrangements were introduced during this period). Furthermore, there was no standardized schedule for follow-up or imaging. In general, patients with advanced disease are followed more closely than patients who have undergone potentially curative treatment. We also did not adjust for treatment. Treatment is strongly correlated with disease stage, each treatment group was very small, there were major changes in treatment policy and the number of available therapies increased rapidly during the period patients were included in the biobank. The influence of treatment is especially relevant for those who have targetable mutations, but not all patients respond to targeted therapy, and there is a large variation in treatment response and response duration for all administered therapies. These differences in diagnostic workup, follow-up and treatment may explain why detection of ctDNA did not remain a significant prognostic factor for PFS in the multivariable analysis, and why there was no statistically significant difference in PFS or OS between the few stage II patients and stage I patients in the multivariable analyses in Table 2, and only a trend towards differences between stage III and stage I patients.

A challenge for any detection technology is the low amount of cfDNA that is available from a plasma sample. This is especially an issue when there is additional loss of DNA in preparation for NGS. We observed a 50% loss in the solutions of constructed cfDNA, and a bigger loss in the patient cfDNA samples (median 78% loss). In contrast, we observed almost no loss in ddPCR. When analyzing the constructed solutions, we observed comparable performance of the NGS approach to ddPCR but cannot rule out that the loss in preparation for NGS led to a lower detection rate than if we had used ddPCR for analyzing the patient samples. The most important reason for using NGS is the ability to detect various mutations in several samples simultaneously. Except for *KRAS* and *EGFR*, only six pairs of patients in our study shared the same point mutation. Furthermore, the cfDNA amount is limited by the available plasma volume. Using an NGS panel enables analysis of several mutations in cfDNA without requiring larger plasma volumes and increases the likelihood of detecting at least one mutation [15]. This was the case for two patients in this study, in which one out of two or three mutations was detected in cfDNA.

Our results add further evidence supporting that detection of tumor mutations in cfDNA is associated with a worse prognosis in NSCLC. A prominent feature of our study is that it suggests that this association is not limited to *EGFR* or *KRAS*. Furthermore, it shows that a small, customized NGS panel may be used for the analysis of cfDNA, which has important implications for feasibility in routine clinical practice. The use of a customized NGS panel increases the sensitivity of detecting ctDNA and reduces the risk of false positives, but the requirement of analyzable

tumor samples limits the use of this approach. Larger prospective clinical trials are necessary to fully explore the clinical value of cfDNA analyses, and several other issues need to be addressed; in general, there is a lack of standardized methods for cfDNA analyses, plasma collection/processing/storage, data interpretation and definition of relevant mutations. Finally, the mechanisms explaining why ctDNA is a negative prognostic factor should be explored.

In conclusion, we found that detectable ctDNA was a negative prognostic factor in NSCLC patients with various tumor mutation spectrums.

CRediT authorship contribution statement

Anine Larsen Ottestad: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Hong Yan Dai:** Conceptualization, Methodology, Writing – original draft. **Tarje Onsøien Halvorsen:** Formal analysis, Writing – review & editing. **Elisabeth Fritzke Emdal:** Investigation, Resources, Writing – review & editing. **Sissel Gyrid Freim Wahl:** Investigation, Writing – review & editing. **Bjørn Henning Grønberg:** Conceptualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A.L.O.: the institution has received grants from AstraZeneca, grants from Roche, during the conduct of the study, H.Y.D.: none T.O.H.: personal fees from Pfizer, personal fees from Pierre Fabre, outside the submitted work, E.F.E.: none, S.G.F.W.: none, B.H.G.: personal fees from MSD, grants and personal fees from AstraZeneca, personal fees from Debiopharm, personal fees from Pfizer, personal fees from Janssen-Cilag, personal fees from Sanofi, personal fees from Takeda, personal fees from Novartis, grants and personal fees from Roche, personal fees from Pierre Fabre, outside the submitted work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2021.100471.

References

[1] N.H. Hanna, A.G. Robinson, S. Temin, et al., Therapy for stage IV non–small-cell lung cancer with driver alterations: ASCO and OH (CCO) joint guideline update, J. Clin. Oncol. 39 (9) (2021) 1040–1091, https://doi.org/10.1200/JCO.20.03570.

[2] N.H. Hanna, B.J. Schneider, S. Temin, et al., Therapy for stage IV non–small-cell lung cancer without driver alterations: ASCO and OH (CCO) joint guideline update, J. Clin. Oncol. 38 (14) (2020) 1608–1632, https://doi.org/10.1200/JCO.19.03022.

[3] P. Goldstraw, K. Chansky, J. Crowley, et al., The IASLC lung cancer staging project: proposals for revision of the TNM stage groupings in the forthcoming (eighth) edition of the TNM classification for lung cancer, J. Thorac. Oncol. 11 (1) (2016) 39–51, https://doi.org/10.1016/j.jtho.2015.09.009.

[4] M. Stroun, J. Lyautey, C. Lederrey, A. Olson-Sand, P. Anker, About the possible origin and mechanism of circulating DNA apoptosis and active DNA release, Clin. Chim. Acta Int. J. Clin. Chem. 313 (1–2) (2001) 139–142, https://doi.org/10.1016/s0009-8981(01)00665-9.

[5] K. Michaelidou, C. Koutoulaki, K. Mavridis, et al., Detection of KRAS G12/G13 mutations in cell free-DNA by droplet digital PCR, offers prognostic information for patients with advanced non-small cell lung cancer, Cells. 9 (11) (2020) 2514, https://doi.org/10.3390/cells9112514.

[6] M. Peng, Q. Huang, W. Yin, et al., Circulating tumor dna as a prognostic biomarker in localized non-small cell lung cancer, Front. Oncol. 10 (2020), https://doi.org/10.3389/fonc.2020.561598.

[7] K. Mardinian, R. Okamura, S. Kato, R. Kurzrock, Temporal and spatial effects and survival outcomes associated with concordance between tissue and blood KRAS alterations in the pan-cancer setting, Int. J. Cancer 146 (2) (2020) 566–576, https://doi.org/10.1002/ijc.32510.

[8] Wahl S.G.F., Dai H.Y., Emdal E.F., et al. Prognostic value of absolute quantification of mutated KRAS in circulating tumour DNA in lung adenocarcinoma patients prior to therapy. J. Pathol. Clin. Res.. Published online January 27, 2021:cjp2.200. doi: 10.1002/cjp2.200.

[9] The Cancer Genome Atlas Research Network, Comprehensive genomic characterization of squamous cell lung cancers, Nature 489 (7417) (2012) 519–525, https://doi.org/10.1038/nature11404.

[10] The cancer genome atlas research network. comprehensive molecular profiling of lung adenocarcinoma, Nature 511 (7511) (2014) 543–550, https://doi.org/10.1038/nature13385.

[11] C. Fiala, E.P. Diamandis, Utility of circulating tumor DNA in cancer diagnostics with emphasis on early detection, BMC Med. 16 (1) (2018), https://doi.org/10.1186/s12916-018-1157-9.

[12] E. Zulato, I. Attili, A. Pavan, et al., Early assessment of KRAS mutation in cfDNA correlates with risk of progression and death in advanced non-small-cell lung cancer, Br. J. Cancer 123 (1) (2020) 81–91, https://doi.org/10.1038/s41416-020-0833-7.

[13] A. Pavan, A.B. Bragadin, L. Calvetti, et al., Role of next generation sequencing-based liquid biopsy in advanced non-small cell lung cancer patients treated with immune checkpoint inhibitors: impact of STK11, KRAS and TP53 mutations and co-mutations on outcome, Transl. Lung Cancer Res. 10 (1) (2021) 202–220, https://doi.org/10.21037/tlcr-20-674. Jan.

[14] T. Ohira, K. Sakai, J. Matsubayashi, et al., Tumor volume determines the feasibility of cell-free DNA sequencing for mutation detection in non-small cell lung cancer, Cancer Sci. 107 (11) (2016) 1660–1666, https://doi.org/10.1111/cas.13068.

[15] B.R. McDonald, T. Contente-Cuomo, S.-J. Sammut, et al., Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer, Sci. Transl. Med. 11 (504) (2019) eaax7392, https://doi.org/10.1126/scitranslmed.aax7392.