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Measuring *KRAS* Mutations in Circulating Tumor DNA by Droplet Digital PCR and Next-Generation Sequencing

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Abstract

Measuring total cell-free DNA (cfDNA) or cancer-specific mutations herein has presented as new tools in aiding the treatment of cancer patients. Studies show that total cfDNA bears prognostic value in metastatic colorectal cancer (mCRC) and that measuring cancer-specific mutations could supplement biopsies. However, limited information is available on the performance of different methods. Blood samples from 28 patients with mCRC and known *KRAS* mutation status were included. cfDNA was extracted and quantified with droplet digital polymerase chain reaction (ddPCR) measuring *Beta-2 Microglobulin*. *KRAS* mutation detection was performed using ddPCR (Bio-Rad) and next-generation sequencing (NGS, Ion Torrent PGM). Comparing *KRAS* mutation status in plasma and tissue revealed concordance rates of 79% and 89% for NGS and ddPCR. Strong correlation between the methods was observed. Most *KRAS* mutations were also detectable in 10-fold diluted samples using the ddPCR. We find that for detection of *KRAS* mutations in ctDNA ddPCR was superior to NGS both in analysis success rate and concordance to tissue. We further present results indicating that lower amount of plasma may be used for detection of *KRAS* mutations in mCRC.

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Introduction

Circulating cell-free DNA (cfDNA) present in blood and other body fluids has initiated a new era in cancer diagnosis and treatment. Apoptotic and necrotic cells from the entire body is the primary source of cfDNA (reviewed by Thierry et al. [1]), and total levels of cfDNA are found to be increased in various cancers [2]. Circulating tumor DNA (ctDNA) is the fraction of cfDNA originating from tumor cells, and ctDNA analyses enable detection of tumor-specific mutations in cfDNA. ctDNA has been estimated to contribute to 0.01%-93% of the total cfDNA [3,4], and ctDNA has been suggested to reflect tumor burden [5–9]. Therefore, ctDNA analysis has been proposed as a noninvasive strategy for gaining insights into the tumor's mutational profile.

Of particular interest is detection of *KRAS*, *BRAF*, and *NRAS* mutations in metastatic colorectal (mCRC) patients, as these are associated with intrinsic resistance to the anti-EGFR antibody treatments offered to this patient group (reviewed by Misale et al. [10]). Concordance rates of 80%-96% between *KRAS* mutations identified in ctDNA analyses and biopsies have been observed [6,11–18]. Next-generation sequencing (NGS) and polymerase chain reaction (PCR)–based techniques for ctDNA analysis are continuously being refined to accommodate the variety of mutations and the low frequencies. Several different approaches have been investigated to accommodate the increasing request for a clinically applicable

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method for ctDNA analysis [19,20], but information on their performance on clinical material remains insufficient.

The aim of the present study was to evaluate and compare methods used for measuring *KRAS* mutations in ctDNA from mCRC patients. Further, in a clinical setting, sample material can be sparse. Therefore, the present study also undertook the aim of investigating the feasibility of analyzing ctDNA from smaller plasma volumes.

Materials and Methods

Patient Samples

Plasma samples originate from a Danish cohort of mCRC patients treated with irinotecan/cetuximab and everolimus according to a closed clinical trial (NCT01387880). Primary data are to be published elsewhere. The *KRAS* tissue genotype was recovered from patient files, but no other clinical data were used. *KRAS* tissue-genotype analysis was performed in a routine setting using Therascreen DXS *KRAS* mutation kit covering codon 12 and 13 (Qiagen, Hilden, Germany). Blood samples were centrifuged at 2300*g* for 15 minutes at 4°C. Plasma was collected and stored at −80°C until further use.

Two subcohorts were used for the current study. Cohort 1 included 28 plasma samples from patients with known *KRAS* mutations in tissue (mutation in codon 12 or 13). Cohort 2 included 16 plasma samples containing at least 2.2 ml plasma and randomly chosen among the available samples. Each sample was divided into a 2 ml and a 200 µl portion, and extraction was performed on these separately. Samples were used to study how extraction of cfDNA from two different volumes affects the total cfDNA measurements. cfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) from a range of 1.5-2 ml plasma for cohort 2 (see Supplementary Table 1) and from either 2 ml or 200 µl for cohort 2 following manufacturer's protocol. Samples were eluted in 100 µl (for 1.5-2 ml plasma samples) or 50 µl (for 200 µl plasma samples) elution buffer (supplied with the kit). cfDNA was frozen at −80°C until further analysis.

After extraction of cfDNA, a 10-fold dilution was made from each sample in cohort 1. Original and diluted samples were used for evaluating how dilution of samples affected the total cfDNA measurements and how the Bio-Rad ddPCR assays performed.

Droplet Digital PCR

Droplet digital PCRs (ddPCRs) were performed using 2X ddPCR Supermix for Probes (no UTP, Bio-Rad), relevant assay (Bio-Rad), and 5 µl cfDNA in a total reaction volume of 20 µl following manufacturer's recommendations. ddPCRs were performed using the QX200 Droplet Digital PCR System (Bio-Rad). Data analyses were performed as recommended by the manufacturer using the QuantaSoft Software version 1.7.4.

Total cfDNA was quantified using an assay targeting *beta-2-microglobuline (B2M)*. Lymphocyte contamination of total cfDNA was detected using an immunoglobulin gene specific assay (*PBC)* as previously described [21]. The assays were multiplexed on the ddPCR platform. Information on assays for B2M and PBC quantification can be found in Supplementary Table 1.

The Bio-Rad *KRAS* PrimePCR ddPCR Mutation assays (Bio-Rad) were used for ddPCR (see Supplementary Table 2). The limit of detection for the assays was determined as recommended by Milbury and colleagues [22].

ddPCR analyses was performed in triplicates. Data from triplicates were merged in the QuantaSoft software and were used for further analysis. Erroneous wells were not included in the merge.

Next-Generation Sequencing

Libraries were prepared using the Oncomine Solid Tumor DNA kit on 1.1-10 ng of cfDNA following manufacturer's instructions (Thermo Fisher Scientific). Amplicon sizes may be found in Supplementary Table 2. Sample concentrations were estimated from the *B2M* measurements. NGS was performed using the Ion Chef Instrument and Ion Personal Genome Machine (PGM) System (Thermo Fisher Scientific). Sequencing was performed using Ion 316 v2 BC chips with eight cfDNA samples per chip.

Primary data processing was performed using the Torrent Suite Software (version 5.0.4) on a Torrent Server and the Ion AmpliSeq Colon and Lung Cancer panel v2 template (Thermo Fisher Scientific). Variant calling was performed using the Ion Reporter Software (version 5.0) and the AmpliSeq CHPv2 peripheral/CTC/CF DNA single sample workflow (Thermo Fisher Scientific). Reference and hotspot BED files were replaced with those supplied with the kit. Default settings were used. Sequencing was considered successful if the mean sequencing depth was ≥2000. If this criterion could not be met, the sample was disqualified. Called variants were only accepted if AF ≥1%. For visualization and manual inspection of variants, the Integrative Genomics Viewer (Broad Institute) was used [23].

The median number of mapped reads per sample was 408,401.5 (range: 233,435-1,414,524), mean depth was 4066.5 (range: 2332-13,438), median uniformity was 100% (range: 94.31-100), and median reads on target were 94.5% (range: 90.3-98.8).

Statistics

All statistics were performed using Stata 13 (StataCorp 2013). Datasets were tested for normality. If data were not normally distributed, log-transformation (using the natural logarithm) was performed to achieve normality, and graphs were produced with log-transformed data. In the *KRAS* mutation analyses, the value 0 was given to wild-type samples. For these datasets, the value 1 was added to all results to enable log-transformation.

For comparing the data produced in this study, the guidelines for comparing methods by Giavarina were used [24]. To test correlation between measurements, linear regression was performed. To test agreement between methods/measurements, Bland-Altman plots and limits of agreement (95% prediction intervals) were used. Results from log-transformed data were back-transformed to achieve meaningful median ratios.

Results

ctDNA Analysis

All cfDNA samples from cohort 1 were sequenced, and the analysis was feasible for 86% of the samples (24 of 28). Among the 24 sequenced samples, there was a 79% (19 of 24) concordance between tissue and cfDNA (see Table 1). For further information on the NGS analysis, see Supplementary Table 4. For samples where the tissue genotype was not confirmed, BAM files were visualized using Integrative Genomics Viewer, and the mutation was identified in three additional samples (10, 11, 12). Common for these was low variant frequency. For sample 26, neither of the mutations detected in tumor were found by NGS ctDNA analysis; instead, a p.Gly12Ala mutation was detected.

Table 1. CtDNA Analysis

Sample ID	Tissue Genotype	NGS	ddPCR-2 ml		ddPCR-200 µl	
		AF (%)	AF (%)	Copies/ml	AF (%)	Copies/ml
1	p.Gly12Val	19.08	22.80	4114.00	22.10	4620.00
2	p.Gly12Ala	37.93	40.40	15708.00	39.80	14300.00
3	p.Gly13Asp	5.51	12.00	607.20	12.20	715.00
4	p.Gly13Asp	19.32	27.20	1848.00	26.50	1969.00
5	p.Gly12Val	1.75	2.40	180.40	1.60	132.00
6	p.Gly12Asp	14.24	26.80	5104.00	25.80	5573.33
7	p.Gly12Val	39.70	60.50	12430.00	60.60	9460.00
8	p.Gly12Ser	1.99	6.00	280.50	7.90	357.50
9	p.Gly13Asp	7.84	16.80	1848.00	15.00	1826.00
10	p.Gly12Ser	0.00	0.70	30.80	0.00	0.00
11	p.Gly12Asp	0.00	1.13	112.20	0.00	0.00
12	p.Gly13Asp	0.00	0.00	0.00	0.00	0.00
13	p.Gly13Asp	23.38	20.60	926.32	16.00	648.42
14	p.Gly12Ala	19.16	26.60	2917.89	25.60	2929.47
15	p.Gly12Val	31.11	44.10	7526.32	43.30	7410.53
16	p.Gly12Val	2.09	2.20	259.11	1.40	158.89
17	p.Gly13Asp [a]	7.55	NA		NA	
18	p.Gly12Asp	NA	2.60	92.40	6.20	198.00
19	p.Gly12Val	NA	15.20	2002.00	13.20	1628.00
20	p.Gly13Asp	20.91	31.30	11088.00	30.40	11550.00
21	p.Gly12Asp	33.36	54.81	255200.00	54.40	242115.80
22	p.Gly12Val	32.27	36.80	35288.00	35.90	33770.00
23	p.Gly12Asp	NA	14.90	1650.00	13.10	1353.00
24	p.Gly12Val	3.56	8.30	682.00	7.80	759.00
25	p.Gly12Val	58.15	66.45	170720.00	NA	
26	p.Gly12Ser	0.00	0.00	0.00	NA	
	p.Gly12Asp	0.00	0.00	0.00	NA	
	p.Gly12Ala [b]	31.68	35.70	40304.00	35.60	39930.00
27	p.Gly13Asp	NA	13.10	875.60	11.10	957.00
28	p.Gly12Asp	0.00	0.00	0.00	0.00	0.00

Abbreviations: AF, allele frequency; ctDNA, circulating tumor DNA; ddPCR, droplet digital PCR; NA, not available; NGS, next-generation sequencing.

[a] This mutation was revealed as a c.38_39delGCinsAT and not the expected c.38G>A by NGS. The mutation was not investigated by ddPCR.

[b] The p.GlyAla was not detected by the tissue genotyping, but exclusively in the ctDNA analysis.

The p.Gly13Asp mutation in sample 17 was revealed as a c.38_39delGCinsAT and not the expected c.38G>A by NGS. Unfortunately, this was not detected in time to allow a change in ddPCR assay, and ddPCR results are therefore unavailable. For the investigated samples, the ddPCR confirmed 89% (24 of 27) of the tissue genotypes (Table 1). The ddPCR analysis confirmed the p.Gly12Ala mutation in sample 26. Further information on the ddPCR analyses can be found in Supplementary Table 4.

Comparison of KRAS Mutation Analyses on ctDNA

Among the 23 samples where both NGS and ddPCR results were available, there was a 91% concordance between the methods (21 of 23, see Table 1). The results obtained from NGS and ddPCR analyses were compared. Three ddPCR analyses were performed on sample 26, and all analyses were included in the comparison. Only samples where methods agreed in either identifying or not identifying the mutation were included (n=23). Good correlation between AFs obtained from the two analyses was obtained (R^2 values of 0.91, see Figure 1*A*). The Bland-Altman analysis presented a mean difference of 6.61 [95% confidence interval (CI): 0.3.69-9.52] and limits of agreement of 5.97-19.18. The 95% prediction interval predicted that measured AFs in NGS versus ddPCR could vary, but the AFs did correlate to those of the NGS analysis.

KRAS Mutation Detection in Small-Volume Samples

KRAS mutation detection was investigated in the diluted samples from cohort 1 to determine if small-volume samples can be used for this purpose. A total of 26 of 28 samples had available material for dilution. We found a 92% concordance with the analysis performed in the original samples (24 of 26, Table 1).

Prior to analysis the diluted samples, we tested how extraction of cfDNA from two different plasma volumes influenced total cfDNA

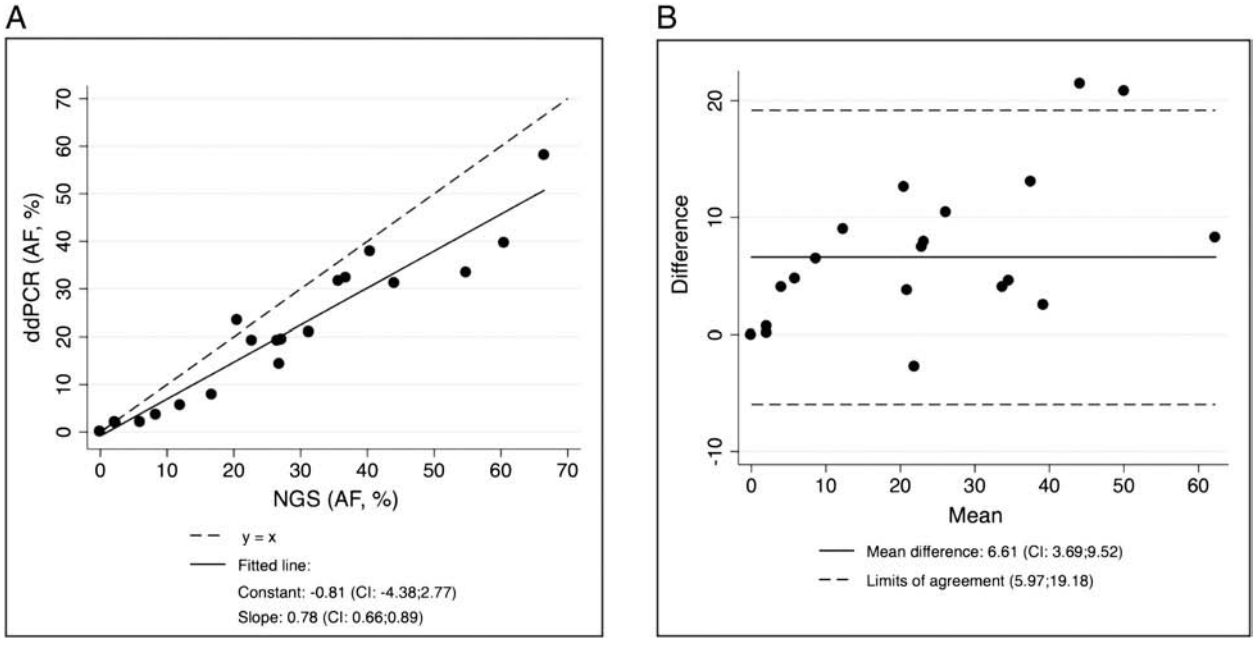

Figure 1. Comparison of the NGS and ddPCR ctDNA analyses. (A) Linear regression from the comparison of NGS and ddPCR AFs (R^2 = 0.91). Results related to the regression are presented below the graph. (B) Bland-Altman plot of the differences (NGS (AF) − ddPCR (AF)).

measurement. The *B2M-PBC* multiplex ddPCR analysis was performed on the samples from cohort 2. Further information can be found in Supplementary Table 5. Three samples were *PBC* positive, and *PBC* exceeded 0.1% of the total cfDNA (see Supplementary Table 5); therefore, these were excluded from further analysis. Comparison of the original and diluted samples resulted in a median ratio (*B2M* 2 ml/*B2M* 200 µl) of 1.15 (95% CI: 1.08-1.22), and limits of agreement were 0.92-1.43, corresponding to a median underestimation of 15% (95% CI: 8-23) of *B2M* in 200 µl samples as compared to 2 ml samples (Figure 2).

Discussion

In the present study, we investigated two different methods, NGS and ddPCR, for investigating *KRAS* mutations in ctDNA. We further investigated the possibility using ddPCR for *KRAS* detection in small-volume samples.

We find that when considering the tissue as gold standard, concordance rates of 79% and 89% for NGS and ddPCR were found. The concordance rates are similar to those found by others [6,11–18]. The deviation between tissue and plasma analyses may be explained by various causes. First, the tissue biopsies were taken at the time of cancer diagnosis, while the plasma samples were collected after at least three lines of palliative chemotherapy. Hence, the mutations found in the tissue biopsy may originate from a clone that has been eliminated by the following treatments [25,26]. Also, new mutations may have arisen during the treatment, explaining our findings in sample 26, where the tissue genotype was not confirmed,but a new *KRAS* mutation was securely detected. Secondly, the NGS analysis only securely detects variants present in ≥1% of cfDNA, while the ddPCR assays have limits of detection ranging between 0.1% and 0.3% (Supplementary Table 2). Studies suggest that the ctDNA fractions are low and highly variable (reviewed by Siravegna et al. [27]), challenging the detection limit of especially the NGS panel used here.

When comparing the results obtained from the ctDNA analyses, we find that ddPCR is superior in identifying the *KRAS* mutations in ctDNA when considering the analysis success rates (NGS: 86%; ddPCR: 100%) and the concordance to tissue genotype. This is in line with other studies [13,28]. When comparing the AFs obtained from the two methods, we find good correlation between them, with a tendency of higher AFs obtained by ddPCR. If a quantitative measure of ctDNA is needed, this finding is relevant since the results may depend on the method used. The best methods for investigating ctDNA and cfDNA are under continuous evaluation (recently reviewed by Sacher et al. [29]). If the mutational tissue status is known and the purpose is to investigate the presence of this mutation in cfDNA, our results suggest that ddPCR is the best method because of the higher success rate, lower time demands, and lower detection limits and costs as compared to the NGS method used here. However, in situations where tissue mutational status is unknown or cannot be recovered, NGS analysis could be preferable for screening the samples. Also, NGS would reveal more information about the constitution of the ctDNA since several genes are often investigated.

Lastly, 92% of the investigated mutations could be recovered in a 10-fold dilution of the plasma samples compared to the full-volume samples. Since the ddPCR reaction is expected to be linear, this may not be surprising. In the present study, extraction of DNA from two different volumes was not feasible in the cohort of patients with known tissue genotype. Instead, we tested extraction on a different cohort where plasma was sufficient. We found that in the extraction of cfDNA from 200 µl plasma, the yield was 15% lower than extraction from 2 ml plasma. This could have influenced our results in the *KRAS* mutation analysis, and the recovery would most likely have been lower (see Supplementary Table 4). For complete elucidation of the extraction efficiency for different input volumes, a larger study is needed.

Despite this, our results indicate that for mCRC low-volume samples may often be sufficient for detection of *KRAS* mutations. This may also be relevant in other solid cancer with high ctDNA shedding.

Conclusion

In this study, we show that the NGS and ddPCR methods investigated have high concordance to tumor genotype (79% and 89%). When comparing the methods, we find that ddPCR is superior

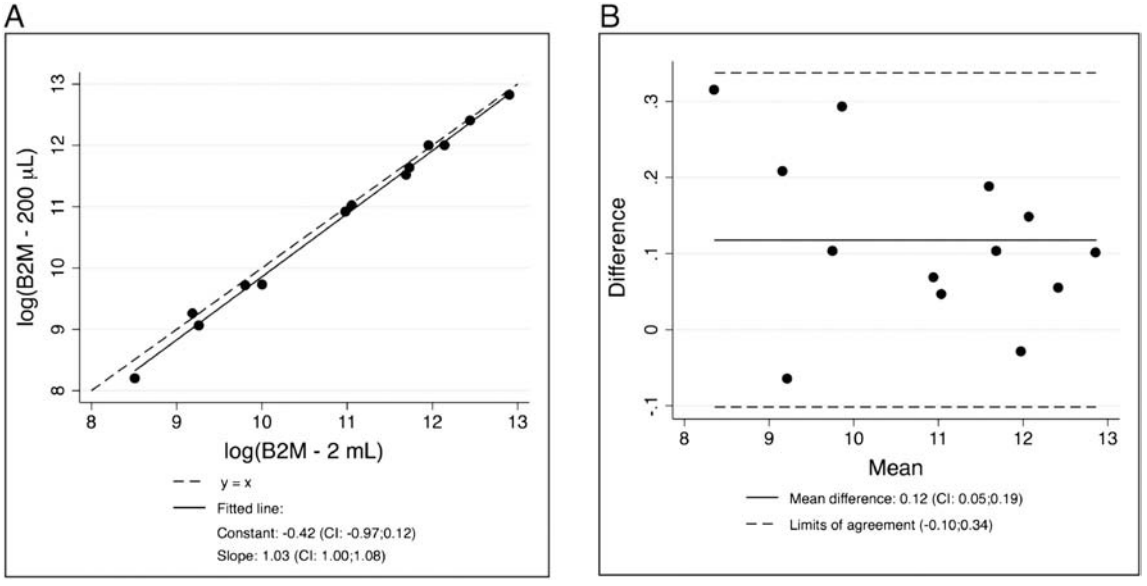

Figure 2. Comparison of log-transformed *B2M* measurements from cohort 2. Constant and slope for the regression and mean difference and limits of agreements are found in the figure. (A) Linear regression on measurements from cohort 2. The regression resulted in an R^2 value of 0.994. (B) Bland-Altman plot of the differences (log(*B2M* 2 ml) − log(*B2M* 200 µl)).

to NGS. And lastly, we find that in cases with sparse material from mCRC patients, smaller plasma volumes may often be sufficient for *KRAS* mutation detection by ddPCR.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.07.013.

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