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Comparison of the quantification of *KRAS* mutations by digital PCR and E-*ice*-COLD-PCR in circulating-cell-free DNA from metastatic colorectal cancer patients

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ABSTRACT

Circulating cell-free DNA (ccfDNA) bears great promise as biomarker for personalized medicine, but ccfDNA is present only at low levels in the plasma or serum of cancer patients. E-*ice*-COLD-PCR is a recently developed enrichment method to detect and identify mutations present at low-abundance in clinical samples. However, recent studies have shown the importance to accurately quantify low-abundance mutations as clinically important decisions will depend on certain mutation thresholds. The possibility for an enrichment method to accurately quantify the mutation levels remains a point of concern and might limit its clinical applicability.

In the present study, we compared the quantification of *KRAS* mutations in ccfDNA from metastatic colorectal cancer patients by E-*ice*-COLD-PCR with two digital PCR approaches. For the quantification of mutations by E-*ice*-COLD-PCR, cell lines with known mutations diluted into WT genomic DNA were used for calibration. E-*ice*-COLD-PCR and the two digital PCR approaches showed the same range of the mutation level and were concordant for mutation levels below the clinical relevant threshold.

E-*ice*-COLD-PCR can accurately detect and quantify low-abundant mutations in ccfDNA and has a shorter time to results making it compatible with the requirements of analyses in a clinical setting without the loss of quantitative accuracy.

1. Introduction

The analysis of circulating-cell-free DNA (ccfDNA) bears great promise as biomarker for personalized medicine and individualized patient management, and technologies for the detection of low-abundance mutations in tumor-derived ccfDNA are currently of great interest [1,2].

Enhanced-*ice*-COLD-PCR is a relatively novel technology that allows the enrichment of any mutation in a region of interest and the subsequent identification using different sequencing approaches [3–5]. However, the possibility to accurately quantify the mutation level by enrichment technologies remains a point of concern and might limit its clinical applicability. For example, for colorectal cancer patients overall and progression-free survival differs in function of the presence of a *KRAS* mutation down to a mutation level of 1%, when treated with an anti-EGFR therapy requiring thus a sensitive detection of the mutation [6]. However, lower levels of mutations do not significantly negatively impact the survival compared to patients with *KRAS* wild-type tumors. Analyses using enrichment methods therefore need to be very carefully controlled using standards with a known level of mutations analyzed in parallel to quantify the mutation level in patient samples.

In the present study, we compared the quantification of *KRAS* mutations in codon 12/13 in tumor-derived ccfDNA from 29 metastatic colorectal cancer patients by E-*ice*-COLD-PCR with two digital PCR approaches, the gold standard technologies for the quantification of rare mutations. Our results show that for samples with mutation levels until below the clinical threshold, E-*ice*-COLD-PCR yields the same results proving the ability to make clinical important decisions based on quantitative thresholds for enrichment technologies.

Abbreviations: dPCR, digital PCR; ddPCR, digital droplet PCR; E-*ice*-COLD-PCR, Enhanced-improved and complete-coamplification at lower denaturation temperature PCR; ccfDNA, circulating cell-free DNA.

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2. Material and methods

2.1. Sample preparation

Twenty-nine patients from a study on circulating biomarkers in metastatic colorectal cancer (NCT01212510) were included in this study. The study was approved by the institutional review board (Northwest I), and all patients provided written informed consent.

Twenty-nine blood samples were collected in EDTA tubes (4 mL) at various stages of chemotherapy, centrifuged at 2700 × *g* for 20 min. Plasma samples were aliquoted and stored at −80 °C until analysis. ccfDNA was extracted from 1 to 2 mL of plasma using the QIAmp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and quantified by fluorescence [7].

KRAS mutations of the tumor tissue were determined using a SNaPshot® multiplex assay targeting different *KRAS* mutations, as previously described [8]. Tumors have not been previously analyzed. Genotype results were *KRAS* (wild-type ($n = 1$), c.34G>T ($n = 3$), c.35G>A ($n = 16$), c.35G>C ($n = 1$), c.35G>T ($n = 4$), c.37G>T ($n = 1$), c.38G>A ($n = 3$)). The tumor genotype was used for the selection of the digital PCR assay targeting the same mutation, but E-*ice*-COLD-PCRs were performed blinded to the results of the genotyping.

2.2. Digital PCR and digital droplet PCR

ccfDNA was pre-amplified with 9 cycles using the Q5 mastermix (New England Biolabs, Ipswich, MA, USA). dPCR analyses were performed on a QuantStudio™ 3D Digital PCR System (QS3D dPCR; Life Technologies, Carlsbad, CA, USA) and a droplet-based dPCR platform (QX200 ddPCR. Qx200® ddPCR system, Bio-rad, Hercules, CA, USA). PCR primers and probes [9] as well as PCR cycling conditions and reagent compositions have previously been described [7,10]. Each assay analyzed a single mutation, which was previously determined in the tumor tissue. CcfDNA from 5 to 11 healthy controls depending on the assay were used to determine the limit of detection for each of the assays targeting the different *KRAS* mutations for the two dPCR platforms.

2.3. E-ice-COLD-PCR

DNA from five cell lines with known mutations in *KRAS* exon 2 were purchased from the Public Health England culture collection (Salisbury, United Kingdom): A549 (34G>A, homozygote), SW480 (35G>T, homozygote), LS174T (35G>A, heterozygote), RPMI-8226 (35G>C, heterozygote) for codon 12 and DLD-1 (38G > A, heterozygote) for codon 13. Cell line were serially diluted into human genomic DNA (Promega, Lyon, France), which was wild-type for *KRAS* mutations, to make a standard curve using standards with mutation levels of 10%, 8%, 6%, 4%, 2%, 1%, 0.8%, 0.6%, 0.4%, 0.2%, 0.1% and 0% of mutations and determine the limit of detection. Each % of mutation was analyzed in sextuplicates.

E-*ice*-COLD-PCR conditions were 1 × HotStar Taq buffer (Qiagen, Courtaboeuf, France) supplemented with 1.6 mM $MgCl_2$, 200 μM of each dNTP, 2.0 U of HotStar Taq polymerase, 200 nM of forward and reverse (CATTATTTTTATTATAAGGCCTGC and Biotin-CAAAATGATTCTGAATTAGCTGT) primers (TIBMOLBIOL, Berlin, Germany), 2 μM of SYTO9, 10 nM of blocker probe (GCTGTATCGTCAAGGCACTCTTGCCTACG + C + CA + C + C + AGCTCCAACTAC-Phosphate) and 2 ng of ccfDNA in a 25 μL volume [3]. qPCRs were performed on a LightCycler 480 thermocycler (Roche Applied Science, Penzberg, Germany). Cycling conditions included an initial denaturation step for 10 min at 95 °C, followed by 6 cycles of 30 s at 95 °C, 20 s at 60 °C and 10 s at 72 °C, followed by 52 cycles of 20 s at 95 °C, 30 s at 70 °C, 20 s at 85 °C, 20 s at 60 °C and 10 s at 72 °C, followed by a melting curve at 20 acquisitions per degree from 65 to 95 °C and a final cooling step at 40 °C. Each sample was analyzed in tri- or quadruplicates.

qPCR reactions were performed to quantify the samples with high mutation levels (>10%), which have reached saturation after enrichment, using 1 × Roche Sybr qPCR master mix (Roche Applied Science), the same PCR primers and 1 ng of ccfDNA. Each sample was analyzed in du- or triplicates. qPCR cycling conditions included an initial denaturation step for 10 min at 95 °C, followed by 50 cycles of 10 s at 95 °C, 45 s at 60 °C and 15 s at 72 °C, followed by a melting curve at 20 acquisitions per degree from 65 to 95 °C and a final cooling step at 40 °C.

Mutation detection, identification and quantification were performed by pyrosequencing on a Pyromark® Q96 HS (Qiagen, Hilden, Germany). For pyrosequencing, 10 μL of the amplification products and pyrosequencing primer (CTTGTGGTAGTTGGAGC) were used [3]. Pyrosequencing was performed according to standard procedures using PyroMark Gold Q96 Reagents (Qiagen). Pyrograms outputs were analyzed with Pyromark 96 ID software (Qiagen) using the allele quantification mode.

An automated MS Excel Visual Basic application was used to quantify and identify mutations from pyrosequencing data [3]. The mutant allele frequency (i.e. relative frequency of an allele) was expressed as the % of mutation analyzed using the pyrosequencing calibrated by the standard curves of the specific mutation. The average and the standard deviation of the % of mutation were calculated using replicates. In addition, the average % of mutation as measured by E-*ice*-COLD-PCR was also recalculated in function of the number of replicates that detected the mutation. As very rare mutated molecules will not be physically present in each assay due to the resulting random distribution of mutated molecules between reactions, successful amplifications might overestimate the average level of mutation as previously shown [11].

All experiments using E-*ice*-COLD-PCR on patient samples were performed blinded with respect to the patient identity, to the tumor genotype and the dPCR results.

3. Results and discussion

E-*ice*-COLD-PCR reaction, which is a qPCR-based enrichment method, was compared to two digital PCR approaches for the quantification of *KRAS* mutations in ccfDNA from the plasma of patients with metastatic colorectal cancer. E-*ice*-COLD-PCR analysis was performed blinded to tumor genotypes and quantification with the two digital PCR approaches.

Low-abundance (under 10%) *KRAS* mutations in the ccfDNA were quantified by E-*ice*-COLD-PCR using five cell lines with known mutations (c.34G>A, c.35G>T, c.35G>A, c.35G>C or c.38G>A) and 10 nM of the LNA blocker (Fig. 1). As no cell line with a 34G>T mutation was included in the study, samples with a 34G > T mutation were quantified using the mean value of all these standard curves. Standard curves with a blocker concentration of 10 nM were logarithmic because the mutation level rapidly reached saturation after enrichment (Fig. 1). A concenration of 10 nM of blocker probe ensured an optimal enrichment of low-abundance mutations under 1%, which is the clinical relevant threshold of *KRAS* mutations in colorectal cancer [6]. Lowering the concentration of blocker to 5 nM improved the linearity of the standard curve especially for higher mutation levels, but reduced sensitivity for low-abundance mutations (data not shown).

Furthermore, samples with a high level of mutations (>10% after calibration) were quantified by a qPCR assay without any blocker. Standard curves of these conventional qPCRs, confirmed that the percentage of detected mutation was linear to the dilution of the fraction of the mutated cell line into the WT genomic DNA (data not shown), but samples with a mutation level below 5% were not detected using this standard qPCR assay.

The E-*ice*-COLD-PCR and two digital PCR methods showed very similar quantitative results with correlation coefficients between 0.94 and 0.98 (Fig. 2). The two digital PCR approaches correlated slightly better ($R^2 = 0.98$), but E-*ice*-COLD-PCR using standard equipment and 96-well formats showed only slightly lower correlations ($R^2 = 0.94$ and $R^2 = 0.95$, Fig. 2). Overall, E-*ice*-COLD-PCR tended to give slightly higher

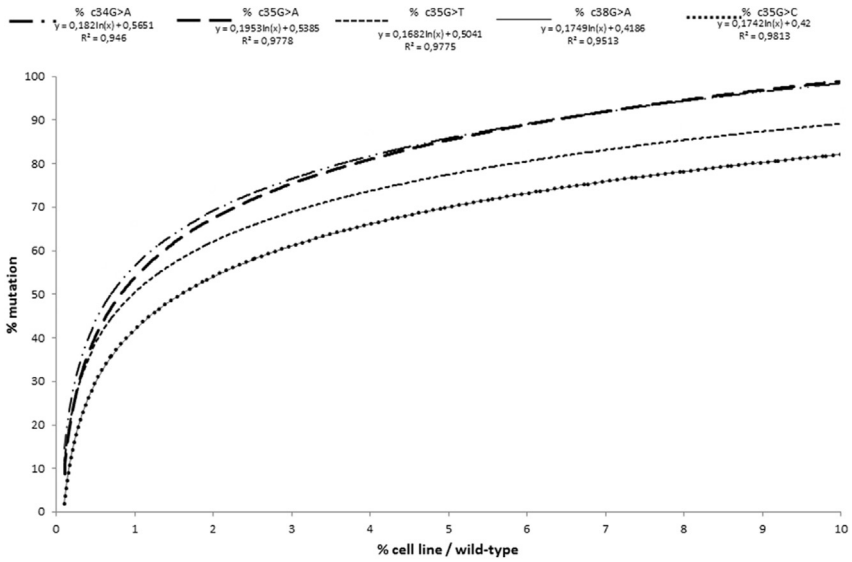

Fig. 1. Quantification by E-*ice*-COLD-PCR using 10 nM of blocker probes and standard curves of five cell lines (A549, SW480, LS174T, RPMI-8226 and DLD-1) with known *KRAS* mutations (c.34G>A, c.35G >T, c.35G>A, c.35G>C or c.38G>A, respectively) diluted in wild-type DNA to obtain mutation levels of 10%, 8%, 6%, 4%, 2%, 1%, 0.8%, 0.6%, 0.4%, 0.2%, 0.1% to 0%. All experiments were performed in sextuplicates. Standard curves were obtained by fitting data on a logarithmic scale and coefficients of regression were calculated for each mutation.

mutation levels compared to the dPCR approaches. Importantly, all technologies showed the same range of the mutation degree and the three technologies were concordant down to a mutation level of ~0.4% corresponding to a statistical average of 2.7 mutated molecules per E-*ice*-COLD-PCR reaction (Fig. 2).

No mutations were detected in ccfDNA sample 21, using these methods which confirmed the WT genotype determined in the tumor DNA. No mutated molecules by either technology were found in samples 1, 9, 24 and 28 despite the presence of a *KRAS* mutation in the tumor. Three of the four samples corresponded to samples, which were collected after several rounds of chemotherapy (3–6). Previous studies have shown that mutations in ccfDNA can be detected in ~80% of patients with known mutation status in the tumor [12,13]. Furthermore, although no information of patient outcome was available for

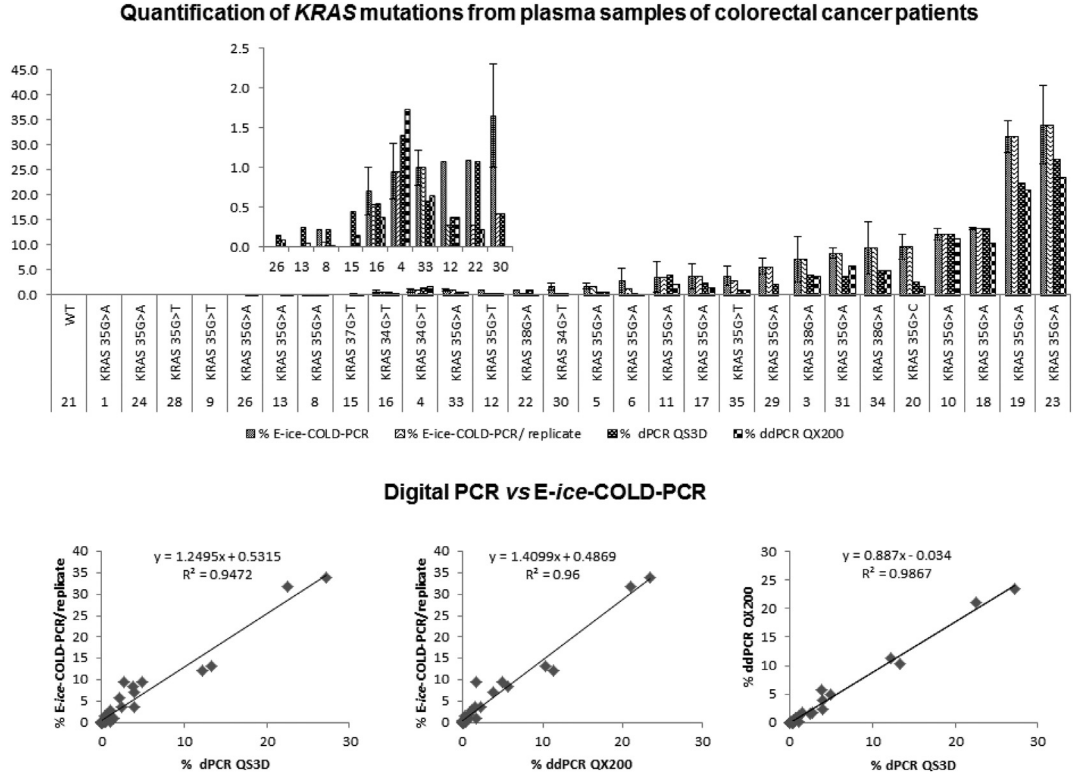

Fig. 2. Comparison of the three quantification methods for *KRAS* codon 12/13 in ccfDNA from colorectal cancer patients using dPCR QS3D, ddPCR QX200 and E-*ice*-COLD-PCR. For each sample, the genotype of the tumor DNA as determined by genotyping is shown. For E-*ice*-COLD-PCR the mean percentage of the detected mutation and % of the detected mutation/replicate are shown as for very low concentrations of mutated molecules not each assay will contain sufficient mutated molecules as previously shown [11]. The quantitative results for all samples are shown in three scatterplots, comparing E-*ice*-COLD-PCR and dPCR QS3D, E-*ice*-COLD-PCR and ddPCR QX200 or the two digital PCRs, respectively. They demonstrate a high linear correlation of the quantification obtained by the three technologies.

the current study, an overall and mutation-specific decrease of ccfDNA has been shown to correlate with better response to chemotherapy [12,14]. It could thus be speculated that the absence of the mutation correlates to an at least temporary success of the chemotherapy. Analyses of additional samples from the patients will be required to evaluate this possibility. In addition, the E-*ice*-COLD-PCR did also not detect reliably low-abundant mutations in samples 26 and 13 because they were below the limit of detection of E-*ice*-COLD-PCR, which was slightly higher compared to digital PCR. However, these mutations were close to the limit of detection in at least one of the digital PCR approaches and while for the second method, the two samples showed the lowest mutation level among the analyzed samples. Finally, sample 15 showing a c.37G>T mutation was not detected using E-*ice*-COLD-PCR, which might be due to the absence of a mutation-enriching LNA at this position, which was not included in the design of the blindly-performed study.

4. Conclusion

We demonstrate here that E-*ice*-COLD-PCR yields highly similar results compared to two dPCR approaches, which can considered as the current gold standard technologies for the detection of rare mutations, and can be used to accurately quantify low-abundant mutations in ccfDNA.

E-*ice*-COLD-PCR provides a sequence-based read-out identifying the occurring mutation instead of a fluorescent signal corresponding to a certain genotype. Furthermore, E-*ice*-COLD-PCR is less costly compared to digital PCR, requires only standard laboratory equipment, can be performed at high-throughput in 96-well plates and has a shorter time to results (~3 h) making it compatible with the requirements of analyses in a clinical setting without the loss of quantitative accuracy. Furthermore, dPCR analyses require either prior knowledge on the mutation present analyzing for example the genotype of the tumor or multiple assays have to be performed to analyze all possible mutations at a mutation hotspot while E-*ice*-COLD-PCR enriches and identifies all mutations at the analyzed mutation hotspot in a single reaction.

There are number of applications with clinical relevance for E-*ice*-COLD-PCR assays [4]. E-*ice*-COLD-PCR can be used to detect mutation in the tumor or in ccfDNA for diagnosis of the disease and might be a useful tool for screening of frequently occurring mutations in populations at high-risk of developing or progressing to cancer. Furthermore, the approach allows detecting mutations at low-abundance that can have an impact on treatment decisions. More importantly and similar to other qPCR, FACS or NGS-based approaches monitor the abundance of mutations in ccfDNA during personalized patient management, which has been shown to correlate and anticipate success or failure of therapy [15–17]. For a subset of patients close to the threshold, digital PCR could complement the results of the E-*ice*-COLD-PCR to strengthen an accurate decision on optimal patient management, but this effort can be concentrated on few individuals only.

Disclosure/conflict of interest

None of the authors has a potential conflict of interest.

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