Clinica Chimica Acta 465 (2017) 1–4

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

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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ARTICLE INFO

Article history:
Received 23 October 2016
Received in revised form 2 December 2016
Accepted 5 December 2016
Available online 7 December 2016

Keywords:
Digital PCR
E-*ice*-COLD-PCR
Circulating cell-free DNA
Mutation enrichment
Quantification
KRAS
Colorectal cancer

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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http://dx.doi.org/10.1016/j.cca.2016.12.004

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