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Screening for circulating RAS/RAF mutations by multiplex digital PCR

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ABSTRACT

Recent years have shown a large interest in the application of liquid biopsies in cancer management. Circulating tumor DNA (ctDNA) has been investigated for potential use in treatment selection, monitoring of treatment response, and early detection of recurrence. Advances have been hampered by technical challenges primarily due to the low levels of ctDNA in patients with localized disease and in patients responding to therapy.
The approach presented here is a multiplex digital PCR method of screening for 31 mutations in the KRAS, NRAS, BRAF, and PIK3CA genes in the plasma. The upper level of the limit of blank, which defines the specificity of the multiplexes, was 0.006%–0.06%. Mutations found by multiplex analyses were identified and quantified by duplex analyses. The method was tested on samples from cholangiocarcinoma patients with known tumor mutational status. Mutations found in the tumor were also found in plasma samples in all cases with analyses for all other mutations being negative. There was a perfect agreement as to wild type status in tumor and plasma.
The method combines a high sensitivity with the ability to analyze for several mutations at a time and could be a step towards routine clinical application of liquid biopsies.

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1. Introduction

Most malignant tumors harbor somatic mutations as an important biological characteristic. The development of malignancy is an ongoing process with continuous development of new mutations, which increases their number in advanced tumors. Somatic mutations occur at a negligible rate in normal cell populations and therefore exquisitely represent specific biomarkers of malignancy [1]. Some of the mutations are bystanders with no or minimal influence on tumor behavior, but a small fraction of them is of crucial importance to proliferation, invasion, and metastatic potential [2].

The analysis of mutations has witnessed an enormous progress during the recent years, especially based on Next Generation Sequencing (NGS). The improved technology, however, has not been reflected as a proportional progress in the clinical application. One reason is tumor heterogeneity. Different parts of the tumor harbor different mutations and it is difficult to identify an overall picture of the clinical relevance. Furthermore, the spectrum of mutations may differ between primary tumor and metastases [3,4]. Another obstacle is the time factor. Analysis of the primary tumor does not necessarily reflect the picture in local or distant recurrence years later. The same problem arises during therapy. Treatment may lead to clonal selection and/or development of new mutations as demonstrated in the treatment of patients harboring a RAS wildtype tumor with monoclonal antibodies against the epidermal growth factor receptor (EGFR) [5–8].

Analysis of plasma or serum may reflect the overall mutational status at the time of therapeutic interest. Most malignant tumors shed mutated DNA into the circulation [5], and the "liquid biopsy" has recently seen considerable scientific and clinical interest. However, there are a number of obstacles on the way to clinical routine use. Mutated DNA in the circulation is present at a low frequency and valid methods for detection of rare events are required. A practical method should be able to analyze a spectrum of mutations in the same run with a minimum of resources. Two methods are generally used - NGS and digital polymerase chain reaction (dPCR), both of which have advantages and disadvantages. NGS is ideal for screening a large number of hotspots for mutations, but the sensitivity is around 2% [9], the costs are still relatively high, and data analysis is rather complex. Digital PCR has the necessary sensitivity (<0.01%) [9], but requires selection of specific mutations for analysis. In principle, PCR is dedicated to analyze one mutation at a time, but it holds potential for several analyses in the same run. The aim of the present study was to investigate an extension of digital PCR for screening of plasma using multiplex technology.

2. Materials and methods

2.1. Materials

A large pool of residual whole blood genomic DNA (gDNA) from individuals genetically screened for lactose intolerance was used as wild

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type donor DNA. Positive controls were generated by site-directed mutagenesis as previously described [10]. Mutated PCR fragments were mixed with gDNA with a concentration of approximately 1600 copies/μl (5.3 ng/μl). Plasma samples from cholangiocarcinoma patients with known tumor mutational status were used for testing the method.

2.2. Plasma preparation

Plasma was isolated from 9 ml EDTA-blood samples by centrifugation at 2000g for 10 min within 2 h of collection. Samples were stored at −80 °C until use.

2.3. DNA purification

DNA from 300 μl of whole blood was purified on a Maxwell 16 purification robot using the Maxwell 16 Blood DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was eluted in 300 μl of the supplied buffer and stored at −20 °C.

Prior to purification plasma was centrifuged at 10,000g for 10 min and the supernatant transferred to clean tubes. 5 μl of internal exogenous spike-in DNA fragment (CPP1) was added to each 1.2 ml of plasma [11]. DNA was purified from 4 × 1.0 ml plasma on the QIAsymphony SP instrument using the QIAsymphony DSP Virus/Pathogen midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted in 4 × 110 μl of the supplied buffer and pooled.

2.4. qPCR

20 μl of pooled DNA from plasma was mixed with 30 μl of water and analyzed by qPCR for the number of gB2M and CPP1 alleles as described previously [11].

2.5. Pre-amplification

The remaining 420 μl of pooled DNA from plasma was concentrated to 20 μl on a Millipore centrifugal filter unit (Millipore, Billerica, MA, USA). The DNA was pre-amplified 10 cycles with Q5 mastermix (New England Biolabs, Ipswich, MA, USA) and pre-amp primer mix (Primers from DNA Technology, Aarhus, Denmark, Suppl. Table 1) in 50 μl reaction volumes according to the manufacturer's recommendations. Cycling conditions were 98 °C for 10 min, 10 cycles of 98 °C for 15 s, 55 °C for 1 min, and 72 °C for 1 min followed by 99.9 °C for 10 min.

2.6. Selection of mutations for multiplex panel

Mutations included in the panel were selected based on publications with data from large series of colorectal tumors sequenced by NGS or pyrosequencing to obtain reliable frequencies of mutations in the KRAS and NRAS genes [12,13]. Mutations found in >0.2% of colorectal tumors were included in the panel. Mutations found at a lower frequency (i.e. mutations found in only one or two patients) were not included. On this basis, 17 mutations in KRAS, 9 mutations in NRAS, and 1 mutation in BRAF were selected. As the significance of PIK3CA mutations is still debated, the four most common mutations in this gene were included in the panel.

2.7. Multiplex set-up

Standard 96-well plate is the format used for droplet digital PCR. Assays were combined in eight multiplex reactions with assays detecting 3–5 mutations in each multiplex (Table 1). All assays detecting mutations in the same or adjacent codons were included in the same multiplex except for KRAS codons 12/13. The specific mutation present in a sample cannot be identified from multiplex analysis. Positive reactions must be split into single reactions to identify the one present.

Table 1
Mutations included in multiplex 1–8.

Multiplex 1	KRAS	Exon 3	Q61H A>C
	KRAS	Exon 3	Q61H A>T
	KRAS	Exon 3	Q61R
	KRAS	Exon 3	Q61L
Multiplex 2	KRAS	Exon 2	G12R
	KRAS	Exon 2	G13C
	KRAS	Exon 4	K117N A>C
	KRAS	Exon 4	K117N A>T
Multiplex 3	KRAS	Exon 4	A146T
	KRAS	Exon 4	A146V
	KRAS	Exon 4	A146P
Multiplex 4	NRAS	Exon 2	G12D
	NRAS	Exon 2	G12C
	NRAS	Exon 2	G12V
	NRAS	Exon 2	G13D
	NRAS	Exon 2	G13R
Multiplex 5	NRAS	Exon 3	Q61K
	NRAS	Exon 3	Q61R
	NRAS	Exon 3	Q61H
	NRAS	Exon 3	Q61L
Multiplex 6	KRAS	Exon 2	G12D
	KRAS	Exon 2	G12V
	KRAS	Exon 2	G13D
	BRAF	Exon 15	V600E
Multiplex 7	KRAS	Exon 2	G12A
	KRAS	Exon 2	G12C
	KRAS	Exon 2	G12S
Multiplex 8	PIK3CA	Exon 9	E542K
	PIK3CA	Exon 9	E545K
	PIK3CA	Exon 20	H1047R
	PIK3CA	Exon 20	H1047L

2.8. Droplet digital PCR – multiplex analysis

Samples were diluted 50 times after pre-amplification. 12 μl of DNA was combined with 5 μl (multiplex 3 and 8) or 12 μl (multiplex 1, 2, 4, 5, 6 and 7) of multiplexes of PrimePCR ddPCR assays for mutations (Bio-Rad®, Hercules, CA, USA; Suppl. Table 2) and Bio-Rad® 2× ddPCR supermix for probes in a total volume of 48 μl. Twenty-two microliter samples were added to two wells of an Eppendorf Twin-tec 96-well plate. Droplets were generated from 20 μl of sample in an Auto Droplet Generator (Bio-Rad®) and 40 cycles of PCR amplification were carried out (95 °C for 10 min, 40 cycles of 94 °C for 30 s, and 55 °C for 60 s followed by 98 °C for 10 min). Samples were analyzed in a Droplet Reader QX100 (Bio-Rad®) and data analyzed with Quantasoft ddPCR Software ver. 1.7.

2.9. Droplet digital PCR – duplex analysis

Samples identified as positive by multiplex dPCR were analyzed by duplex dPCR for assays included in that multiplex. The procedure was as described for multiplex dPCR and the same dPCR assays used (Suppl. Table 2). Five microliters of duplex assay was used for each 48 μl reaction volume. Twenty microliters of sample was analyzed per well.

2.10. Controls

Negative controls (water and gDNA) were pre-amplified and analyzed simultaneously when samples were pre-amplified. Positive controls were included in digital PCR analysis at every mastermix preparation.

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