DROPLET-BASED MICROFLUIDICS FOR THE QUANTITATIVE DETECTION OF RARE MUTATIONS

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ABSTRACT

Somatic mutations within tumoral DNA can be used as highly specific cancer biomarkers. The use of gene alterations as biomarkers in clinical oncology requires the detection of the tumor specific changes within a large excess of non-mutated DNA from normal cells. The majority of genetic tests that identify variations of DNA sequences incorporates a step of PCR. However, this method has a limited sensitivity when amplifying complex mixtures of DNA. We describe a procedure combining droplet-based microfluidics, emulsion PCR and recent advances in molecular diagnostic for the highly sensitive quantitative detection of mutated DNA within complex mixtures of DNA.

KEYWORDS: Droplet-based microfluidic, digital PCR, highly sensitive detection, *KRAS*, biomarkers.

INTRODUCTION

Gene alterations associated with an increased risk of developing specific cancers have been highlighted. By allowing characterization of a tumor at the molecular level, such **biomarkers** are especially important for diagnosis and treatment of cancer. Cancer biomarkers can be classified in three types: prognostic (treat or not treat?), predictive (which drug?) and pharmacodynamics (what dose?), each of which can help on the development of efficient therapeutic strategies and anti-cancer drugs[1].

From the many possible biomarkers ***KRAS*** constitutes a good model since it can be used to detect cancer but also as a predictive tool to guide the decision of whom to treat with a specific treatment. Indeed, mutations in *RAS* gene can be found in about 30% of all human tumours with *KRAS* being the most frequently mutated member of this gene family. *KRAS* has been found mutated in adenocarcinomas of the pancreas (70–90% incidence), colon (50%) or lung (25–50%)[2]. Moreover, the presence of *KRAS* mutations is associated with an absence of response to colorectal cancer targeted therapies employing anti-EGFR antibodies[3]. However, recent research has highlighted the necessity for highly sensitive detection methods to detect mutated *KRAS* genes in cells within the highly genetically heterogeneous tissues that constitute tumors[4].

THEORY

The use of gene alterations as biomarkers in clinical oncology requires a highly sensitive strategy that allow the detection of the tumor specific changes in a background of non-mutated DNA from normal cells[5]. However, the majority of genetic tests which aim to identify variations of DNA sequences incorporate a step of PCR and this method has a limited sensitivity when amplifying complex mixtures of DNA (like DNA extracted from tumors, plasma or feces). The best current strategies present a minimal threshold of detection of mutation of around 0.1% which greatly restricts their applications. However "**emulsion PCR**" or ePCR[6], can overcome these limitations. This **high-throughput digital PCR** procedure is based on dividing a normal PCR mixture between the aqueous droplets of a water-in-oil emulsion such that there is, in most cases, not more than one template DNA molecule per droplet[7,8]. This method permits each DNA to be amplified separately in a droplet rather than amplifying a pool of different DNAs (Figure 1).

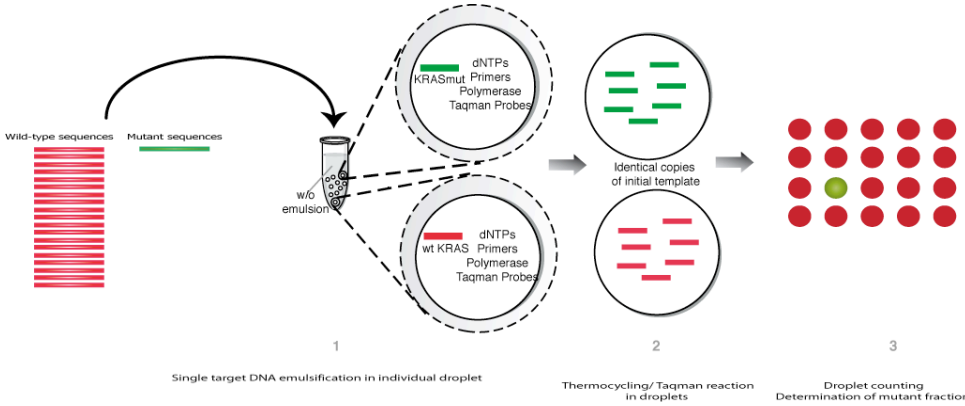

Figure 1*. Taqman reaction in droplet. Template DNA is encapsulated so that each droplet does not contain more than one target DNA molecule in the presence of specific Taqman probe(s) targeting specific DNA sequence(s) and all component allowing the PCR to be performed (1); during amplification of the targeted sequence(s), the Taqman probes is cleaved and its fluorophore released. In this example, the amplification of a wild-type sequence will release a green fluorophore when the amplification of a mutant sequence will release a red fluorophore; the number of green and red droplets is determined giving the proportion of mutant and wild type DNA in the tested sample (3).*

We describe a method which combines ePCR and recent advances in PCR-based diagnosis. Thanks to the segregation

of individual target DNA-molecules in billions of aqueous droplets acting as independent microreactors, this procedure allows extremely **precise**, **sensitive** and fast **quantification** of mutated genes to be carried out. The sensitivity of the method is limited only by the number of droplets that can be analyzed: screening a million droplets allows 1 mutated DNA molecule in a background of a million of non-mutated DNA molecules to be detected (one template DNA per droplet). This non-invasive method allowing the **quantitative detection** of a minority of mutated sequences within a large excess of wild-type sequences and the precise determination of the proportion of the targeted DNA(s) (mutated biomarker(s)) in the initial samples. Another specific and unique feature of the method is its ability to perform multiplex analysis allowing detection of several different mutations within a single biomarker, mutations in different biomarkers, or even to perform whole targeted pathway analysis.

EXPERIMENTAL

Taqman reaction in aqueous droplets.

Genomic DNAs were extracted from the cell-line SW48 bearing wild-type *KRAS* alleles (ATCC CCL-231) and the heterozygotous cell-line LoVo (LoVo (ATCC CCL-229) bearing a mutant allele of the *KRAS* oncogene (G12C) and a wild-type allele. The TaqMan® probes and the primers (Applied Biosystems) allowing amplification of the target sequences used in this study were previously validated for clinical sample analysis[3]. The probes specific for mutated sequences are conjugated to 6-FAM fluorophore (λ_{ex} 494nm/λ_{em} 522nm) and the probes complementary to wild-type sequence bear NED (λ_{ex} 546nm/λ_{em} 575nm) fluorophore.

Emulsification of PCR mixtures using microfluidic systems.

Microfluidic chips were fabricated by patterning channels in poly(dimethylsiloxane) (PDMS) using conventional soft lithography methods[9] as described previously[10] to produce droplet size of 10pL. The oil phase consisted of HFE-7500 (3M) fluorinated oil containing 3% (w/w) of EA surfactant (RainDance Technologies, Lexington, MA), a PEG-PFPE amphiphilic block copolymer[11]. Droplets were generated by flow-focusing[12]. The samples were collected in a home-made collection/reinjection device (see Figure 2) and thermocycled for amplification by PCR.

Fluorescent signal detection.

Samples were analysed by spreading the emulsion between two 0.17-mm-thick microscope coverslips. Images were aquired with a Zeiss (Jena, Germany) LSM510 laser-scanning confocal microscope equipped with C-Apochromat 20× (n.a. 0.8) water immersion objectives. 6-FAM fluorophore was excited at 488 nm using an argon laser, and emission was recorded from 505 to 550 nm. Images were processed with the Zeiss LSM Image Browser software, version 2.50.0929. The emulsion can also be reinjected into specific microfluidic devices and the fluorescence of each droplet measured using a previously described optical set-up[10].

RESULTS AND DISCUSSION

In our procedure, see Figure 2, target DNAs are individually compartmentalized in microdroplets using microfluidic systems and the PCR-based assay can be performed in a classic PCR apparatus (in a collection/reinjection vial or a PCR tube). After thermocycling, the droplets are reinjected or analyzed directly on a glass slide to measure fluorescence (Figure 2, Panel 4, left).

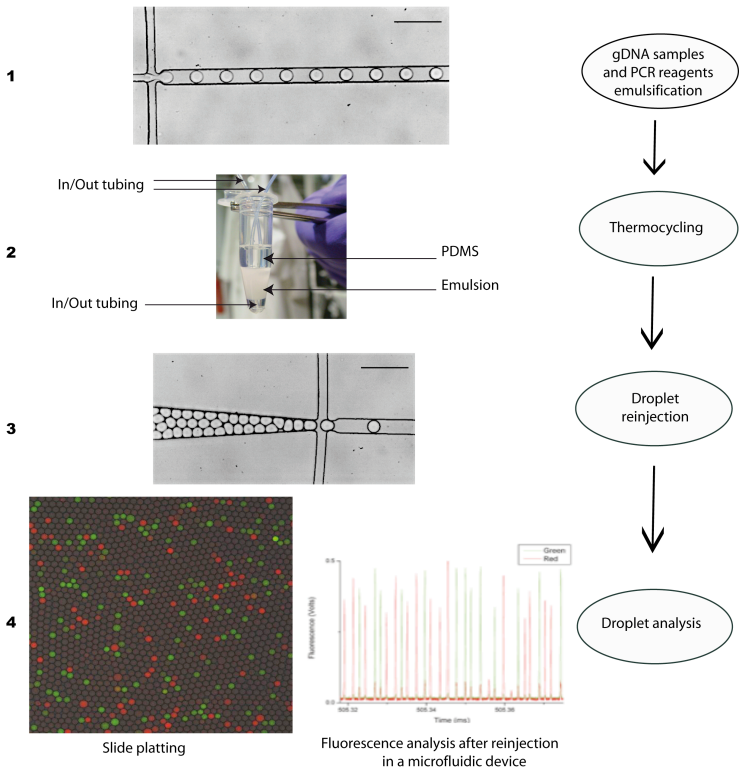

Figure 2: Overview of the procedure. *(1) PCR mix (containing the probes, sample DNA and all components allowing the PCR reaction to be performed) is emulsified within a microfluidic device (the straight black line represents 100 micrometers). (2) The emulsion is collected in a specific PDMS-sealed tube that is thermocycled allowing the fluorogenic reaction to occur. (3) The emulsion is then dispersed on a glass slide and the droplets are imaged globally (4, left) or reinjected in a microfluidic chip with appropriate fluorescence set-up (4, right). Time sequence on the right corresponds to reinjection of droplets containing 6-FAM or NED fluorophores. The micrograph on the left corresponds to the fluorescence analysis of thermocycled droplets containing genomic DNA extracted from LoVo cells. Bright green droplets correspond to droplets containing mutant DNA, bright red droplets correspond to droplets containing wild-type DNA and other droplets do not contain any targeted DNA.*

Dual probe Taqman assays were used to demonstrate accurate quantification of the mutated *KRAS* oncogene within samples made of fragmented genomic DNA containing a large excess of the wild-type oncogene. The sensitivity of the procedure was tested up to 1/200,000 dilution of genomic DNA containing mutant allele in genomic DNA bearing the wild-type oncogene (8% of the droplets contained a *KRAS* allele in that particular experiment). Our experiments clearly demonstrated that the sensitivity of the procedure is only limited by the number of droplets being analyzed. Moreover, we have also demonstrated the quantitative specific detection of the seven most common mutations of the *KRAS* oncogene in a background of non mutated DNA within a single experiment.

CONCLUSION

We have developed an innovative non-invasive procedure for the screening of specific cancer biomarkers, which can be easily used in diagnosis, prognosis or prediction settings in the clinical management initially of colorectal cancer patients and then extended to other type of cancers. We propose an original quantitative method that will allow to reach, at a moderate cost, an unprecedented sensitivity. In addition, instead of a 'yes or no' readout typically resulting from commonly used procedures, we will be able to accurately quantify the proportion of mutated DNA in a tested samples. The method should find multiple applications ranging from diagnosis, cancer recurrence monitoring or treatment management. Various patient samples could be tested using this procedure, including tumor, plasma, feces or urine. The proposed strategy allows extremely precise, sensitive tests to be performed compared to existing procedures. By allowing experiments to be performed that are impossible using existing technologies, our method set a new standard for cancer diagnosis.

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