MULTIPLEX HIGHLY SENSITIVE DETECTION OF CANCER BIOMARKERS IN BIOLOGICAL SAMPLES

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ABSTRACT

Gene alterations within tumoral DNA can be used as highly specific biomarkers to distinguish cancer cells. These DNA biomarkers are especially important for the diagnosis, prognosis, treatment and follow-up of patients. In order to have the required sensitivity and specificity to detect rare tumoral DNA in patient samples, a simple, sensitive and quantitative procedure to measure the ratio of mutant to wild-type genes is required. This paper reports a new procedure allowing the highly sensitive multiplex detection of biomarkers within genomic DNA extracted from Cancer Cell-lines.

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

INTRODUCTION

Complex networks of genetic alterations have been highlighted for most cancers. These somatic mutations are present in tumor cells but not in normal cells and can serve as highly specific **biomarkers**.[1] They represent molecular signatures of cancer cell phenotype and thus constitute great tools for **early cancer detection** as well as **prognostic assessment**.[2] Moreover, **target-oriented treatments** have met increasing developments in the last decade and new biomarkers of resistance or sensitivity to such treatment have been highlighted.[3] Plain use of these Biomarkers in clinical oncology requires highly sensitive and specific methods to detect them accurately in clinical samples. Mutations in *KRAS* are amongst the most common oncogenic alterations in a range of human cancers. 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%).[4] The *KRAS* oncogene is constitutively activated by a small set of specific mutations which almost all occur in codons 12 and 13 of exon 2.[5]

THEORY

Plain use of gene alterations as biomarkers in clinical oncology requires a highly sensitive, and ideally quantitative, strategy that allow the detection of the tumor specific modifications in a background of non-mutated DNA from normal cells.[6] Techniques such as dual probe TaqMan® assays and pyrosequencing classically used in clinics, while quantitative, cannot detect less than ~1% mutant genes in a background of non-mutated DNA from normal cells.[7] Indeed, 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).

Digital techniques however, by allowing the analysis of many individual DNA molecules in parrallel rather than analysing a pool of different DNAs, allow more precise and sensitive quantification of mutated DNA. Digital PCR, which is based on the compartmentalization and amplification of single DNA molecules,[8] allows the discrete counting of the mutant and wild-type alleles present in a sample. Its sensitivity is only limited by the number of molecules that can be analyzed and the false positive rate of the mutation detection assay. **Emulsion PCR** (ePCR)[9] is a **high-throughput digital PCR** procedure 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. Digital PCR can also be performed in aqueous droplets separated by oil in microfluidic systems. In contrast to classical procedures droplet-based microfluidic systems allow the creation of highly monodisperse droplets (<1.5% polydispersity) at kHz frequencies and their precise manipulation (see[10] for a review). Millions of reactions can be analyzed in parallel.

We describe a method which combines digital PCR in microfluidic systems and recent advances in PCR-based diagnosis. By segregating 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. 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 (gDNAs) were extracted from tumoral cell-lines (ATCC, LGC, Molsheim, France): SW48 (ATCC CCL-

231, wild-type DNA), H358 (ATCC CRL-5807, G12C), LS123 (ATCC CCL-255, G12S), SW620 (ATCC CCL-227, G12V), H1573 (ATCC CRL-5877, G12A), and LoVo (ATCC CCL-229, G13D). 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.[11] 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.

Droplet-based microfluidic procedures.

Microfluidic chips were fabricated by patterning channels in poly(dimethylsiloxane) (PDMS) using conventional soft lithography methods as described previously to produce droplet size of 10pL.[12, 13] The oil phase consisted of HFE-7500 (3M) fluorinated oil containing EA surfactant (RainDance Technologies, Lexington, MA), a PEG-PFPE amphiphilic block copolymer.[14] The droplet-based strategies are described in Fig. 1 and 2. Samples were analyzed by spreading the emulsion between two 0.17-mm-thick microscope coverslips. Images were acquired with a Zeiss (Jena, Germany) LSM510 laser-scanning confocal microscope equipped with C-Apochromat 20× (n.a. 0.8) water immersion objectives. Images were processed with the Zeiss LSM Image Browser software, version 2.50.0929. Emulsions were also reinjected into specific microfluidic devices and the fluorescence of each droplet measured using a previously described optical set-up.[15]

RESULTS AND DISCUSSION

Our procedure is based on using a droplet-based microfluidic system to perform digital PCR in millions of picolitre droplets[12]. The gDNA is compartmentalized in droplets at a concentration of less than one genome equivalent per droplet together with two TaqMan® probes, one specific for the mutant and the other for the wild-type DNA, which generate green and red fluorescent signals, respectively. After thermocycling, the ratio of mutant to wild-type genes is determined by counting the ratio of green to red droplets (see Figure 1).

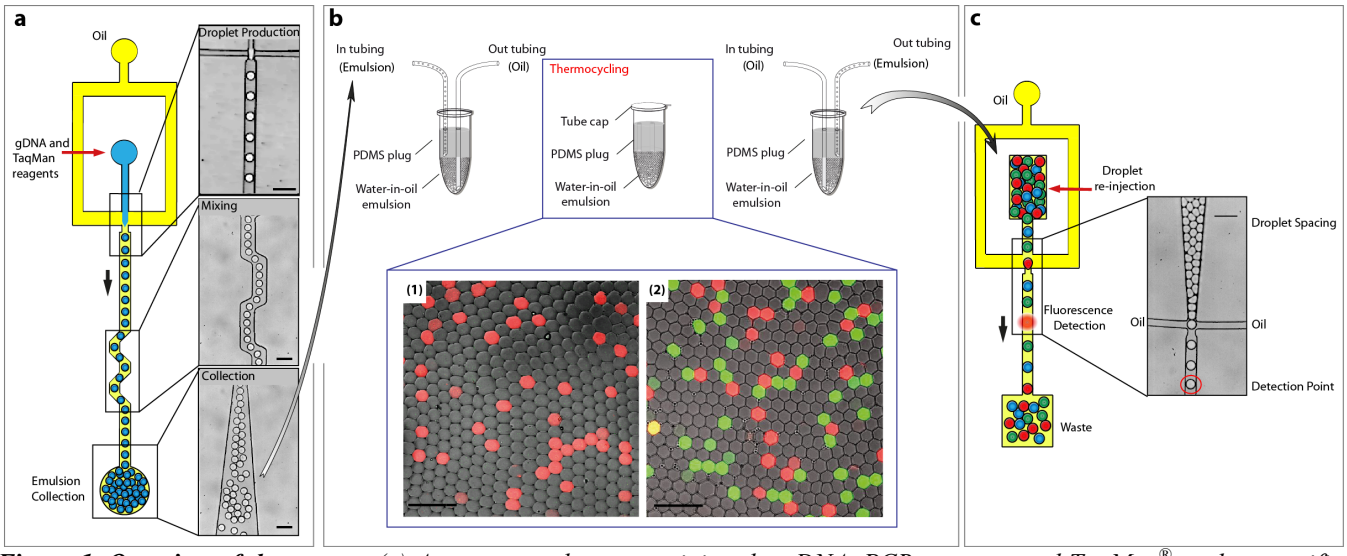

Figure 1. Overview of the system. *(a) An aqueous phase containing the gDNA, PCR reagents and TaqMan® probes specific for the wild-type and mutant genes is emulsified using a microfluidic device. (b) The emulsion is collected in a PDMS-sealed and thermocycled. During DNA amplification, the TaqMan® probes are cleaved and the corresponding fluorophores are released. Light micrographs of drop production, mixing, collection and re-injection are shown (scale bars 60 µm).* ***Middle Panel: Fluorescence confocal microscopy analysis of thermocycled droplets.*** *gDNA extracted from homozygous cell lines bearing wild-type KRAS alleles (1) and a heterozygous cell-line bearing both mutant and wild-type KRAS alleles (2) were analyzed. Red-fluorescent droplets contain wild-type DNA, green-fluorescent droplets contain mutant DNA, yellow-fluorescent droplets contain both mutant and wild-type DNA, and non-fluorescent droplets do not contain target DNA (scale bar 100µm).*

By using this procedure we demonstrated the detection of up to 1/200,000 mutant DNA in wild-type DNA,[13] while using the same probes and classic bulk procedures, clinicians demonstrated a sensitivity of 10%.[11] The sensitivity of the method is limited only by the number of droplets that can be analyzed. We extended our technology to detect and quantify these mutations in a single experiment. First, using a rather simple set-up we demonstrated the detection of less than 1% of a specific mutant DNA within gDNA. In that experiment, an optical encoding procedure is used to identify the mutation present in the biological sample. Second, by using one-to-one fusion of drops containing gDNA with any one of seven different types of droplets, each containing a TaqMan® probe specific for a different *KRAS* mutation, or wild-type *KRAS*, and an optical code, we also demonstrated that it was possible to screen the six common mutations in *KRAS* codon 12 in a single experiment (see Figure 2 for the procedure).

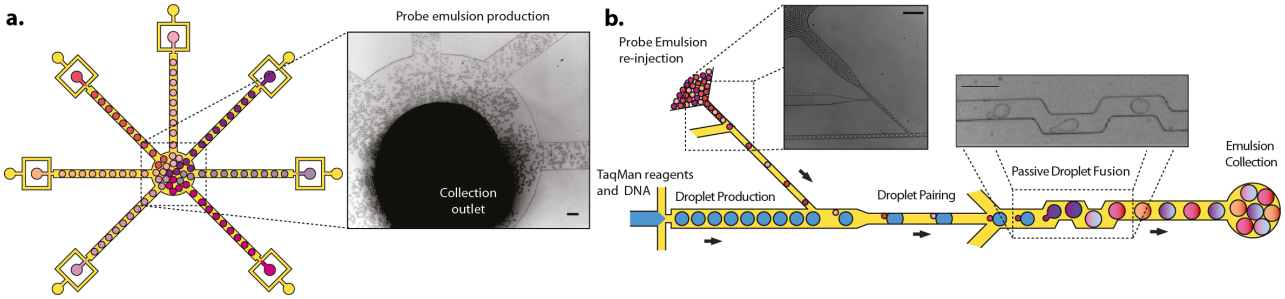

Figure 2. Parallel analysis of multiple mutations. *(a) A device capable of producing seven different types of droplets -each containing a different TaqMan® probe that targeted either the wild-type or one of the six most common mutations in codon 12 of the KRAS oncogene- was used to generate a probe emulsion. In addition, each type of droplet contained a different concentration of a red-fluorescent dye (Dextran Texas Red, DTR). The pooled droplets were collected off-chip and reinjected onto a passive fusion device (b) where they were fused one-to-one with droplets produced on-chip containing all PCR reagents and gDNA. Light micrographs of probe emulsion production and re-injection, as well as droplet fusion are shown (scale bar, 100 µm). The fused emulsion was submitted to the procedure described in Fig. 1.*

CONCLUSION

Such highly sensitive procedure allows a quantitative detection of rare biomarkers, bringing the possibility to search for those mutations in samples such as blood, plasma or feces, Our method could constitute a new paradigm for non-invasive cancer diagnosis. The tools, protocols and procedures presented in this paper can also, be used for appropriate patient management by identifying which patient are more likely to benefit from specific targeted treatment or detecting Cancer recurrence in a precise and non-invasive way within various bodily fluids.

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