QUANTITATIVE DETECTION OF CIRCULATING TUMOR DNA IN PLASMA SAMPLES BY DROPLET DIGITAL PCR.

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ABSTRACT

This paper reports the use of droplet-based digital PCR for detection and quantification of biomarkers in circulating DNA from plasma of patients with advanced colorectal cancer (CRC). Furthermore, an original method is described for differentiating gene alterations within a single test on the basis of fluorescence intensity by varying the concentration of fluorescent probes.

KEYWORDS

Digital PCR, Cancer biomarkers, Droplet-based microfluidics, Microfluidic, Droplets.

INTRODUCTION

Circulating tumor DNA (ctDNA) is present in plasma of individuals with advanced cancers. ctDNA is a prognostic marker for patients with CRC and it might also be used for predicting the response to targeted therapy. Although ctDNA is characterized by the presence of a somatic mutation, direct quantitative detection through a simple workflow of such mutant DNA is not feasible by current technologies because the ratio of ctDNA to wild-type DNA can be as low as 1/10,000. Among potential biomarkers, mutations in the *KRAS* oncogene constitute an ideal target. Mutations in *KRAS* are indeed found mutated at high rates in colon cancer, pancreatic and lung cancer and *KRAS* mutation is a predictive factor of resistance for therapies targeting EGFR. More than 90% of the activating mutations occur at 7 DNA positions in 2 codons (12 and 13).

EXPERIMENT

The procedure, presented in MicroTas 2010 [1], is based on using a droplet-based microfluidic system to perform digital PCR in millions of picolitre droplets [2]. It allows for extremely precise, sensitive, and fast quantification of mutated genes within complex mixtures of DNA. The sensitivity of the procedure was confirmed by measuring 1/200,000 dilution of KRAS-mutated cell-line DNA in a background of wild-type DNA. As a comparison, when using the same probes, PCR in bulk demonstrated a sensitivity of 10%[3]. This procedure was applied to DNA extracted from plasma of patients with metastatic CRC to detect either of the two most frequent mutations of *KRAS* (G12D and G13D) and the wild-type DNA. DNA concentration in the plasma samples varied by two orders of magnitude and was not correlated with the proportion of mutated DNA, which varied from 42% to 0.1%. The expected mutation (known by primary tumor characterization) was detected in 16 out of the 19 samples. Two samples had a low amount of amplifiable DNA leading to an inconclusive result.

Conventional qPCR has limited multiplexing capability due to spectral overlap of fluorescent probes. Multiplexing enables development of assays for biomarker panels at reduced cost and sample consumption, increased throughput and the potential for built-in assay controls. 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, and presented in MicroTas 2011 [4], that it was possible to screen the six common mutations in KRAS codon 12 in a single experiment [5]. In such strategy, a target molecule will be tested for only one type of mutation and rare mutations could be missed. We now developed a new method for differentiating targets on the basis of fluorescence intensity by varying the concentration of the fluorescent probes [6]. To demonstrate, results of a 4- and 5-plex TaqMan® assay for KRAS mutations were measured simultaneously with just VIC and FAM fluorophores (Figure 1a,b).

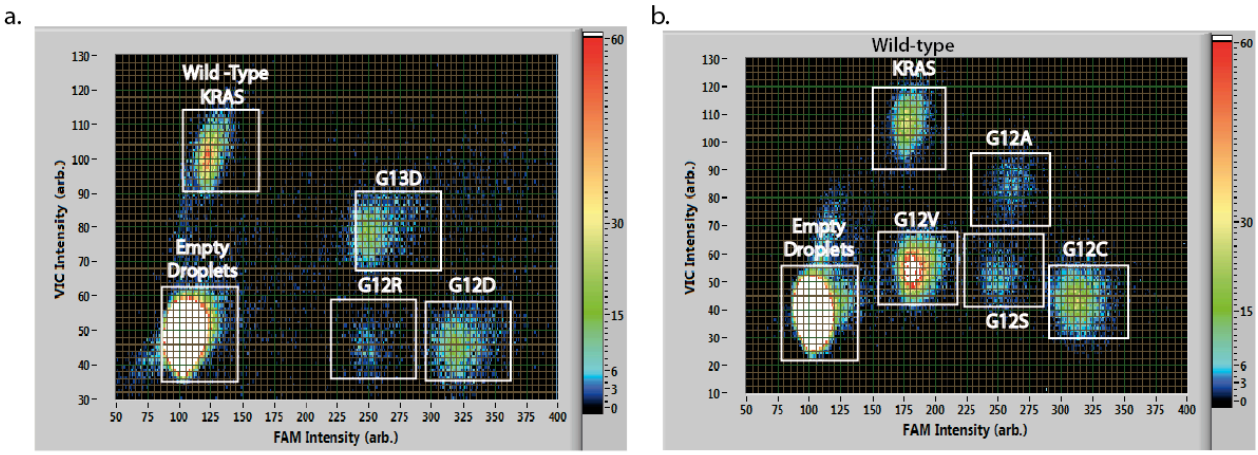

Figure 1: *TaqMan assays for each of the seven most frequent mutations of KRAS were assembled into two multiplex panels by mixing mutation-specific VIC and/or FAM TaqMan probes with a single wild-type (VIC) probe and a single pair of PCR primers in each panel (a and b). The heat-map histograms reveal that concentrations of probes were tuned to enable discrimination of droplets containing no amplifiable fragments, wild-type KRAS DNA, or a DNA fragment with a unique KRAS mutation.*

Sensibility of the procedure for each *KRAS* mutation was demonstrated at up to 1/10,000 (see Figure 2 for an example with G12S and G12V).

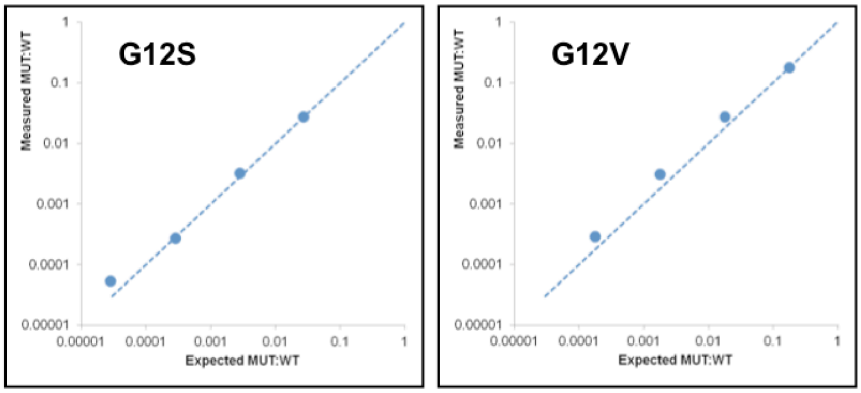

Figure 2: Sensitivity detection of KRAS alleles in the multiplex procedure. *DNA isolated from a homozygotous cell-line for the G12S or G12V mutation was mixed with wild-type DNA to prepare serial dilutions over four decades of mutant-to-wild-type ratio. The samples were analyzed with the appropriate multiplex digital PCR panel a). The results indicate that each mutation is detectable across the range of concentrations.*

Circulating DNAs isolated from plasma of 54 patients with metastatic colon cancer with known *KRAS* status (based on previous tumor characterization) were analyzed with these multiplex assays (Figure 3). Among these samples, 19 were expected to be positive for a KRAS mutation based on previous tumor DNA characterization. After multiplex analysis, 13 samples matched the mutation identified in the tumor DNA, 1 sample contained a mutation different from the one expected from the tumor characterization (annotated as ***). No mutation was detected in 1 plasma sample for which a mutation was expected. The last 4 samples were inconclusive for the expected mutation (2 were positive in duplex analysis). Among the 35 samples that were expected to be negative for a *KRAS* mutation based on previous tumor DNA characterization, 2 plasma samples were positive for a mutation (annotated as * and **). For one of these samples (*), the initial tumor sample contained less than 15% of tumor cells and interestingly the patient had a progressive disease at the first evaluation under cetuximab. The other (**) was not evaluated for the number of tumor cells due to the size of the biopsy.

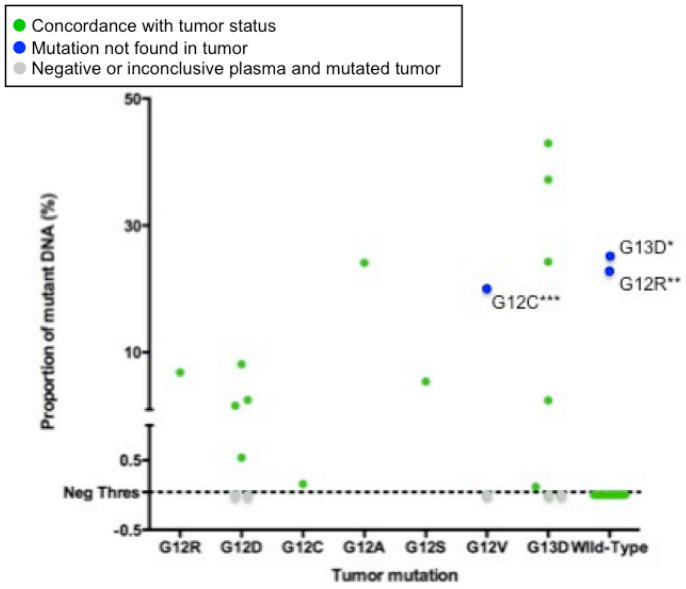

Figure 2: Analysis of 54 plasma samples of patient with advanced cancer by multiplex droplet-based PCR. *The mutational status (mutated for one of the seven most frequent KRAS mutations or non mutated) of the primitive tumor is indicated in the x-axis and the proportion of mutant DNA within the sample in y-axis.*

Our results demonstrate that our digital PCR method enables non-invasive detection of *KRAS* in plasma of patients with metastatic CRC with high sensitivity and specificity. We anticipate that the method will be employed in multiple applications in the clinic, including diagnosis, cancer recurrence monitoring, and treatment management.

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