

DETECTION AND QUANTIFICATION OF MINORITY KRAS SUBCLONES IN TUMORS USING DROPLET-BASED MICROFLUIDICS: CLINICAL IMPLICATION

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ABSTRACT

We used a multiplex droplet-based digital PCR method in a retrospective study including 181 patients with a metastatic colorectal cancer (mCRC) for the quantitative and sensitive detection of *KRAS* and *BRAF* mutations in DNA extracted from tumors.

KEYWORDS: digital PCR (dPCR), *KRAS* mutations, cancer biomarkers, droplet-based microfluidics

INTRODUCTION

Colorectal cancer is the 3rd most common cancer worldwide [1]. 1 million patients are diagnosed each year and 50% of them will eventually develop a metastatic disease [2]. Recent efforts to improve the treatment of metastatic colorectal cancer has led to the development of monoclonal antibodies such as Cetuximab (or its fully humanized version Panitumumab) that inhibit the activation of the Epidermal Growth Factor Receptor (EGFR) and its downstream pathways that promote cell growth, proliferation and the inhibition of apoptosis.

Mutations in the *KRAS* and *BRAF* genes are found at high rates in colon cancers and are correlated with the non-response to anti-EGFR therapies. However, only 50% of patients with apparently non-mutated tumors give an objective response to treatment [3] and for most of these patients a secondary resistance invariably ensues, the cause of which remains poorly understood. The presence of a small subpopulation of cells with mutated genes within the tumor, undetected by conventional procedures can be the cause of these treatment resistances.

THEORY

Up to date the most widely used technique for the assessment of *KRAS* mutational status in clinical samples is the direct sequencing. This technique offers a sensitivity of 20% (mutant alleles represent at least 20% of the total DNA in the sample for the detection to be reproducible) [4]. Most of the studies aimed at the determination of the predictive role of *KRAS* mutations in the response to anti-EGFR therapies used allele-specific PCR (qPCR), which has a sensitivity of 10% [5].

Quantitative procedures with a higher sensitivity for the detection of mutations in the *KRAS* and *BRAF* genes are required in order to determine if the apparently non-mutated tumors are indeed carriers of subpopulations with mutated genes. The required sensitivity can be obtained with the use of digital PCR techniques coupled with microfluidic systems [6].

EXPERIMENTAL

We previously described a picodroplet-based microfluidic technique for the highly sensitive and quantitative detection of *KRAS* mutations in biological samples [7,8] able to detect up to 1/200,000 mutant DNA in wild-type DNA. We subsequently adapted this technique in a multiplexed format for the quantitative detection of the 7 common mutations of *KRAS* (in codon 12 and 13) (Fig.1) and applied it to the detection of circulating tumor DNA in plasma samples from patients with a metastatic colorectal cancer [9].

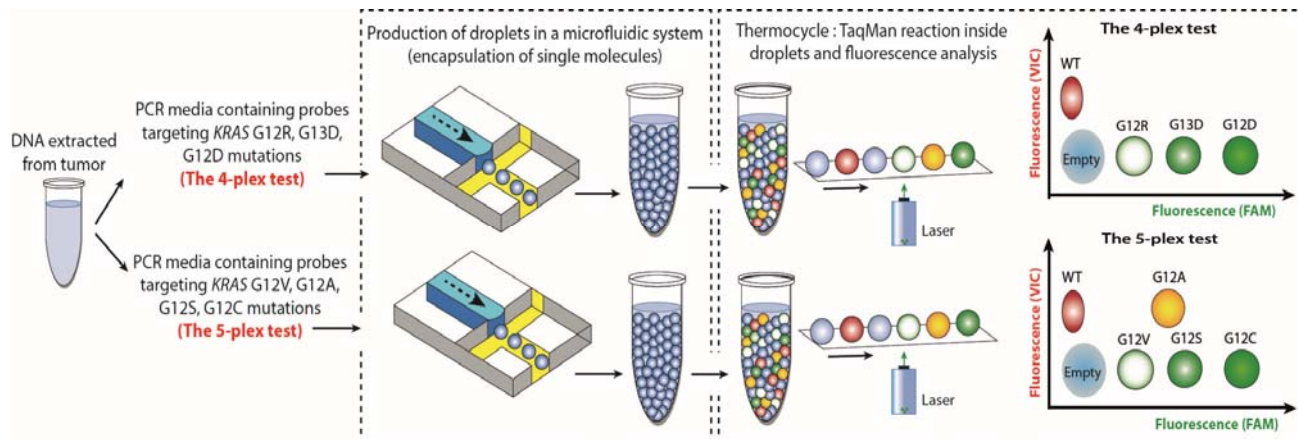


Figure 1: Multiplex detection of the seven most frequent mutations of the *KRAS* gene: TaqMan assays for each of the seven most frequent mutations of *KRAS* (G13D, G12R, G12D, G12V, G12A, G12S and G12C) were assembled in two multiplexed panels by mixing mutation-specific VIC and/or FAM probes in tuned concentrations with a single wild-type specific VIC probe in each panel. The tuning of the probe concentrations allows to discriminate between droplets containing no amplifiable fragments, wild-type *KRAS* DNA or a DNA fragment with a unique *KRAS* mutation.

We now applied this procedure for the screening of DNA extracted from tumors of patients (108 men, 73 women with 62 years of age \pm 11) with a metastatic disease, all refractory to conventional chemotherapy (Irinotecan) and treated with anti-EGFR combined with Irinotecan or as monotherapy.

RESULTS AND DISCUSSION

Tumors of 43 patients were already characterized as bearing a *KRAS* mutation by conventional TaqMan analysis. These were detected as positives in droplets with proportions of mutated DNA from 11 to 64% (mean of 38.2% \pm 16.5%). Within the 138 negative tumors, 23 were found positive in droplets with *KRAS* proportions from 0.01% to 12.4% (mean of 2.1% \pm 3.2%). Among the mutated tumors, additional subclones were detected only in droplets. Thanks to the enhancement of sensitivity provided by the use of droplet digital PCR coupled with microfluidics we were able to observe an inverse correlation between the amount of mutated DNA and the response to anti-EGFR treatment (Fig. 2, $P < 0.0001$). The Progression Free Survival of patients with tumor presenting less than 5% mutated *KRAS* was comparable to those of patients with non-mutated tumors.

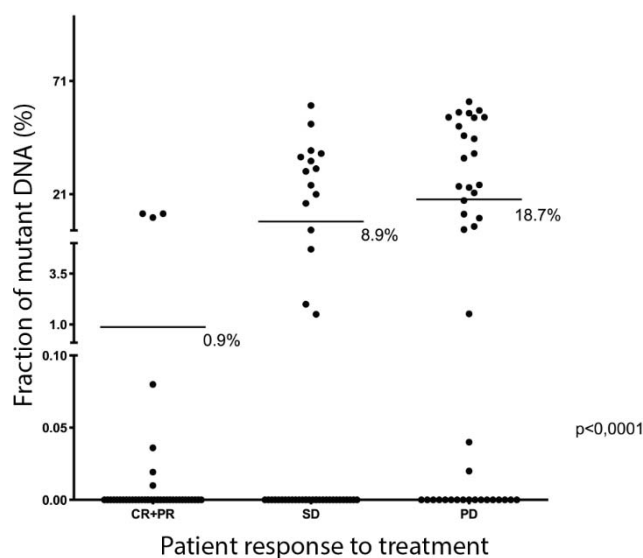


Figure 2: Fraction of mutated DNA for patients who present complete or partial responses (CR and PR); Stable disease (SD) or progressive disease (PD). Response to anti-EGFR therapy correlates with the proportion of mutated DNA in tumors.

CONCLUSION

Our results demonstrate the importance of a quantitative approach for the detection of low frequency *KRAS* mutations within tumor samples for the therapeutic management of patients.

With the quantitativity and the higher sensitivity provided by droplet-based microfluidics, we also show that the amount of mutated DNA is indicative of the response to treatment and the survival of patients.

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REFERENCES:

- [1] K.M. Tveit *et al.*, Phase III trial of Cetuximab with continuous or intermittent Fluorouracil, Leucovorin, and Oxaliplatin (Nordic FLOX) versus FLOX alone in first-line treatment of metastatic colorectal cancer: the NORDIC-VII study. *J Clin Oncol*, vol. 30 no. 15 1755-1762, (2012)
- [2] D. Parkin *et al.*, Global Cancer Statistics, 2002. *CA Cancer J Clin*, vol. 55, Issue 2, 74–108, (2005)
- [3] A. Lièvre, *et al.*, *KRAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab, *J Clin Oncol*, vol. 26, pp. 374-379, (2008)
- [4] J.H. van Krieken *et al.*, *KRAS* mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program, *Virchows Arch*, vol. 453, pp. 417-431, (2008)
- [5] F. Molinari *et al.*, Increased detection sensitivity for *KRAS* mutations enhances the prediction of anti-EGFR monoclonal antibody resistance in metastatic colorectal cancer, *Clin Cancer Res*, vol. 17, pp. 4901-4914, (2011).
- [6] Taly *et al.*, Detecting biomarkers with microdroplet technology, *Trends Mol Med*, vol. 18, pp. 405–416, (2012)
- [7] Pekin, *et al.*, Droplet-based microfluidics for the quantitative detection of rare mutations, 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS), Groningen, Netherlands, p. 58-60, (2010).
- [8] Pekin *et al.*, Quantitative and sensitive detection of rare mutations using droplet-based microfluidics, *Lab Chip*, vol. 11(13), pp. 2156-2166 (2011).
- [9] Pekin *et al.*, Quantitative detection of circulating tumor DNA in plasma samples by droplet digital PCR, 16th International conference on miniaturized systems for Chemistry and Life Science (MicroTas), Okinawa, Japan, p. 169-171 (2012).