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Droplet digital PCR of circulating tumor cells from colorectal cancer patients can predict *KRAS* mutations before surgery

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ABSTRACT

In colorectal cancer (CRC), *KRAS* mutations are a strong negative predictor for treatment with the EGFR-targeted antibodies cetuximab and panitumumab. Since it can be difficult to obtain appropriate tumor tissues for *KRAS* genotyping, alternative methods are required. Circulating tumor cells (CTCs) are believed to be representative of the tumor in real time. In this study we explored the capacity of a size-based device for capturing CTCs coupled with a multiplex *KRAS* screening assay using droplet digital PCR (ddPCR). We showed that it is possible to detect a mutant ratio of 0.05% and less than one *KRAS* mutant cell per mL total blood with ddPCR compared to about 0.5% and 50–75 cells for TaqMeltPCR and HRM. Next, CTCs were isolated from the blood of 35 patients with CRC at various stage of the disease. *KRAS* genotyping was successful for 86% (30/35) of samples with a *KRAS* codon 12/13 mutant ratio of 57% (17/30). In contrast, only one patient was identified as *KRAS* mutant when size-based isolation was combined with HRM or TaqMeltPCR. *KRAS* status was then determined for the 26 available formalin-fixed paraffin-embedded tumors using standard procedures. The concordance between the CTCs and the corresponding tumor tissues was 77% with a sensitivity of 83%. Taken together, the data presented here suggest that is feasible to detect *KRAS* mutations in CTCs from blood samples of CRC patients which are predictive for those found in the tumor. The minimal invasive nature of this procedure

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in combination with the high sensitivity of ddPCR might provide in the future an opportunity to monitor patients throughout the course of disease on multiple levels including early detection, prognosis, treatment and relapse as well as to obtain mechanistic insight with respect to tumor invasion and metastasis.

1. Introduction

The recent development of next-generation sequencing has allowed the identification of key genetic and epigenetic changes in human tumors. This has facilitated the development of a novel group of anticancer agents targeting oncogenic signaling pathways associated with specific mutations (Shen et al., 2015). In colorectal cancer (CRC), genetic alterations are frequent in genes encoding downstream effectors of the epidermal growth factor receptor (EGFR) pathway including *KRAS*, *NRAS* and *BRAF* as well as genes involved in pathway crosstalk like *PTEN* or *PIK3CA* (Therkildsen et al., 2014; Muzny DM et al., 2012). *KRAS* mutations are observed for 40–45% of CRC patients with the 7 most frequent mutations occurring in codons 12–13. These mutations have been identified as a strong negative predictive factor for the response to EGFR-targeted antibodies including cetuximab and panitumumab. Therefore, the detection of *KRAS* status, and more recently, *NRAS* status has become mandatory before starting the treatment of metastatic CRC patients with EGFR-directed antibodies. Previous clinical trials have demonstrated that patients with *KRAS* mutant tumors have marginal or no response to EGFR-directed antibodies whereas up to 40% of patients with *KRAS* wild-type (wt) tumors respond to the treatment (Amado et al., 2008; Lievre et al., 2006). However, the necessity to do *KRAS* genotyping of tumor tissues may be problematic in many clinical situations. For instance, for non-metastatic CRC, most patients are rapidly operated after diagnosis. 25–30% of these patients will develop liver or lung metastasis within 5 years due to the growth of occult micrometastasis. In this case, the *KRAS* status in the primary tumor may no longer be available and serial biopsies of the metastasis is generally not feasible. Furthermore, the *KRAS* status of the primary tumor may not necessarily reflect the current *KRAS* status of the metastasis since tumors evolve during disease progression by both Darwinian selection and therapeutic pressure (Chisholm et al., 2015). The same argument holds for the 25% of patients harboring metastasis at diagnosis. Finally, following tumor removal it is advisable to determine the presence of residual disease and to monitor the efficiency or resistance to therapies (Misale et al., 2014; Bird et al., 2006). For all these reasons, alternative assays to *KRAS* testing of the primary tumor are warranted.

Liquid biopsies including circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) has become an exciting tool in oncology to investigate the dynamics of tumor development over time (Heitzer et al., 2015; Lianidou et al., 2015; Gingras et al., 2015; Ma et al., 2015). Besides ctDNA, one of the most promising applications would be molecular characterization of CTCs. CTCs are rare cells present in the blood and lymphatic vessels which have been released from the primary tumor and/or metastasis and which may have the potential to induce relapse or metastasis (Hardingham et al., 2015; Pantel and Speicher, 2015; Joosse et al., 2014). It is widely accepted that the number of CTCs is inversely correlated with the prognosis of the patients for multiple solid tumors including both metastatic and non-metastatic CRC (Lim et al., 2014; Cohen et al., 2009; Cohen et al., 2008) suggesting that these cells are potentially harmful. At present, relatively little is known about their mutational status because molecular characterization of CTCs requires both a specific method to isolate the CTCs from the hematogenous cells (Alix-Panabières and Pantel, 2014; Pantel and Alix-Panabieres, 2013; Torino et al., 2013; Lianidou, 2014) as well as a molecular detection method with high sensitivity in order to be able to identify a few mutant copies against a strong background of wt DNA (Cai et al., 2015).

First-generation Sanger sequencing is currently the gold standard for detection of mutant DNA, However, it is only reliable for detection of 10–20% mutant copies at the allelic level (Tsiatis et al., 2010). Since lower levels of mutant copies would be masked by a high background of wt DNA from contaminant leukocytes, Sanger sequencing would not be appropriate for molecular characterization of CTCs. Other PCR-based approaches for detecting rare mutant copies have been developed, all with advantages and disadvantages in terms of cost, sensitivity and specificity. This includes pyrosequencing (Tsiatis et al., 2010), co-amplification at lower denaturation temperature-PCR (cold-PCR) (Zuo et al., 2009), amplification refractory mutation system using a bifunctional self-probing primer (Franklin et al., 2010), massively-parallel sequencing (Peeters et al., 2013), high-resolution melting (HRM) analysis (Simi et al., 2008; Mohamed Suhaimi et al., 2015) or allele specific blocker PCR (ASB-PCR) (Mohamed Suhaimi et al., 2015). Recently, droplet digital PCR (ddPCR) (Hindson et al, 2011, 2013) has been developed to detect very low number of mutant copies. This system partitions the PCR products into approximately 20,000 droplets, each of which undergoes an individual reaction. Each individual droplet is defined on the basis of fluorescent amplitude as being either positive or negative. This technique has been successful for characterization of hotspot mutations in ctDNA (Hudecova, 2015; Bettegowda, 2014) including the detection of *KRAS* mutations (Thierry et al., 2014). Recent studies have explored the feasibility to detect *KRAS* mutant cells by ddPCR for the diagnosis of pancreatic cancer (Earl et al., 2014) or other type of mutations such as Estrogen-receptor-1 in metastatic breast cancer (Guttery, 2015) thereby opening new avenues for molecular characterization of CTCs.