MOLECULAR ONCOLOGY 10 (2016) 1575–1584

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases

Nick Beije[a,*], Jean C. Helmijr[a], Marjolein J.A. Weerts[a], Corine M. Beaufort[a], Matthew Wiggin[b], Andre Marziali[b], Cornelis Verhoef[c], Stefan Sleijfer[a], Maurice P.H.M. Jansen[a], John W.M. Martens[a]

[a]Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands
[b]Boreal Genomics, Vancouver, BC, Canada
[c]Department of Surgical Oncology, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form
4 August 2016
Accepted 3 October 2016
Available online 10 October 2016

Keywords:
Circulating tumor DNA
Cell-free DNA
Metastatic colorectal cancer
Somatic mutations
Next-generation sequencing

ABSTRACT

Assessing circulating tumor DNA (ctDNA) is a promising method to evaluate somatic mutations from solid tumors in a minimally-invasive way. In a group of twelve metastatic colorectal cancer (mCRC) patients undergoing liver metastasectomy, from each patient DNA from cell-free DNA (cfDNA), the primary tumor, metastatic liver tissue, normal tumor-adjacent colon or liver tissue, and whole blood were obtained. Investigated was the feasibility of a targeted NGS approach to identify somatic mutations in ctDNA. This targeted NGS approach was also compared with NGS preceded by mutant allele enrichment using synchronous coefficient of drag alteration technology embodied in the OnTarget assay, and for selected mutations with digital PCR (dPCR). All tissue and cfDNA samples underwent IonPGM sequencing for a CRC-specific 21-gene panel, which was analyzed using a standard and a modified calling pipeline. In addition, cfDNA, whole blood and normal tissue DNA were analyzed with the OnTarget assay and with dPCR for specific mutations in cfDNA as detected in the corresponding primary and/or metastatic tumor tissue. NGS with modified calling was superior to standard calling and detected ctDNA in the cfDNA of 10 patients harboring mutations in *APC*, *ATM*, *CREBBP*, *FBXW7*, *KRAS*, *KMT2D*, *PIK3CA* and *TP53*. Using this approach, variant allele frequencies in plasma ranged predominantly from 1 to 10%, resulting in limited concordance between ctDNA and the primary tumor (39%) and the metastases (55%). Concordance between ctDNA and tissue markedly improved when ctDNA was evaluated for *KRAS*, *PIK3CA* and *TP53* mutations by the OnTarget assay (80%) and digital PCR (93%). Additionally, using these techniques mutations were observed in tumor-adjacent tissue with normal morphology in the majority of patients, which were not observed in whole blood. In conclusion, in these mCRC patients with

Abbreviations: ctDNA, circulating tumor DNA; cfDNA, cell-free DNA; mCRC, metastatic colorectal cancer; dPCR, digital PCR; IonPGM, Ion Personal Genome Machine.

* *Corresponding author*. Wytemaweg 80, 3015 CN, Rotterdam, The Netherlands. Fax: +31 10 7041005.
E-mail address: n.beije@erasmusmc.nl (N. Beije).

http://dx.doi.org/10.1016/j.molonc.2016.10.001

oligometastatic disease NGS on cfDNA was feasible, but had limited sensitivity to detect all somatic mutations present in tissue. Digital PCR and mutant allele enrichment before NGS appeared to be more sensitive to detect somatic mutations.

1. Introduction

The use of targeted therapies has markedly transformed cancer treatment in the last decade (Haber et al., 2011). Unfortunately most of the responses to targeted therapies in the advanced setting are transitory at best, because intrinsic or acquired resistance to these agents is present or rapidly develops (Leto and Trusolino, 2014). Tumor heterogeneity is thought to play a pivotal role in the development of acquired resistance (Turner and Reis-Filho, 2012). Heterogeneity is present in the tumor lesion itself (intra-tumor heterogeneity), while in the advanced setting also heterogeneity between different metastatic lesions (inter-metastatic heterogeneity) can be present (Gerlinger et al., 2012; Vogelstein et al., 2013). Furthermore, during effective treatment the genomic landscape of tumor cells evolves. For example, there are strong indications that the emergence of *KRAS* mutations in metastatic CRC (mCRC) patients who initially harbored a tumor wildtype for *KRAS*, contributes to resistance against anti-EGFR monoclonal antibodies (Misale et al., 2012; Siravegna et al., 2015). Altogether, this clearly stresses that in the advanced setting, particularly after treatment with agents dependent on a genetic aberration, the analysis of a single biopsy to evaluate the cancer genome and to guide treatment decision making is likely insufficient. The only way to acquire a comprehensive overview of the cancer genome would be to take multiple biopsies from metastases, which is cumbersome and even impossible in some patients due to inaccessibility of lesions.

As an alternative approach to taking biopsies from solid lesions, assessing circulating tumor DNA (ctDNA) in the peripheral blood has been proposed as a minimally-invasive way to evaluate the tumor mutation status. Tumor cells release fragmented DNA into the peripheral blood, and these DNA fragments can be detected as ctDNA in the cell-free compartment (i.e., serum and plasma) of the blood. It is thought that ctDNA can represent the most prevalent tumor clones from primary tumors as well as metastatic lesions. In the last years, various techniques have been introduced to detect and quantify mutations in ctDNA. Generally, for choosing a technique to detect mutant ctDNA one has to take into account the rarity of ctDNA alleles relative to wild-type DNA alleles in the cell-free compartment of the blood. Frequencies of ctDNA vary largely, from roughly $<0.1\%$ to $>10\%$ (Diehl et al., 2008; Haber and Velculescu, 2014). Techniques such as digital PCR (dPCR) (Wang et al., 2010) and BEAMING (Diehl et al., 2006) have the advantage of superior sensitivity, being able to detect ctDNA in frequencies as low as 0.01%. However, using these techniques only one or a limited number of specific somatic mutations can be analyzed simultaneously. Recently, a technique called synchronous coefficient of drag alteration (SCODA) (Marziali et al., 2005; Thompson et al., 2012) has been used to develop an assay (OnTarget assay) which is able to analyze up to 96 mutant alleles in 9 genes with reported sensitivity similar to dPCR and BEAMING of 0.01–0.001% (Kidess et al., 2015). This OnTarget assay firstly enriches for mutant alleles and subsequently genes are targeted next-generation sequenced (NGS). Still, when all somatic variants in numerous genes are of interest, NGS for multiple genes is indicated. A potential drawback of these NGS techniques is however their lack of sensitivity for detecting ctDNA frequencies below 1–2% (Diaz and Bardelli, 2014).

The current study set out to explore the feasibility of Ion Torrent PGM (IonPGM) targeted NGS on plasma cfDNA of patients with mCRC undergoing colorectal liver metastasectomy. To this purpose, 12 patients undergoing resection of CRC metastases were investigated. In these patients the primary tumor, the resected liver metastasis, cfDNA and normal tumor-adjacent tissue were sequenced using a 21-gene CRC-specific panel on the IonPGM platform. In addition, to gain more insight into the advantages and disadvantages of ctDNA detection with different techniques, results generated with the IonPGM platform were compared with the OnTarget assay and with digital PCR for specific variants.

2. Methods

2.1. Patients and sample collection

Patients with colorectal liver metastases undergoing resection of liver metastases were included as part of a prospective study in the Erasmus MC Cancer Institute evaluating the prognostic value of circulating tumor cells (CTCs) as described before (Lalmahomed et al., 2015; Mostert et al., 2015). Prior to surgery 30 mL of blood was drawn in EDTA tubes from all patients for DNA isolation from plasma or whole blood as described in the next paragraph. In addition, 30 mL of blood was drawn in CellSave tubes and subsequently processed for CTC enumeration on the CellSearch system (Janssen Diagnostics, Raritan, NJ, USA) as described previously (Lalmahomed et al., 2015). During surgery, the liver metastases and normal tissue of the liver were collected and freshly frozen (FF) for later analyses. In all cases the tissue was also stored as formalin-fixed paraffin-embedded (FFPE). In patients presenting with synchronous CRC metastases, the resection of the colon was combined with the resection of the liver metastases and normal tumor-adjacent colon was also collected.

For this mutation analysis study, only patients with a complete set of available plasma cfDNA, primary tumor tissue, metastatic tumor tissue and normal tumor-adjacent tissue of the liver or the colon were included. Other criteria for inclusion were 1) acquisition of metastatic tissue, normal tissue and plasma on the same day; 2) a minimum percentage of 30% tumor cells in the primary tumor sample and the liver metastasis sample as assessed using hematoxylin and eosin (H&E) slides from macro-dissected tissue by an experienced pathologist; 3) no tumor cells detected in macro-dissected tissue from tumor-adjacent colon and liver as assessed on H&E slides by an experienced pathologist; 4) no adjuvant treatment given in case of metachronous metastases. All patients provided written informed consent and the institutional board approved the protocols (Erasmus MC ID MEC-2006-089).

2.2. DNA isolation

DNA was isolated from all tissues using the NucleoSpin DNA tissue kit (Macherey–Nagel, Düren, Germany). For FFPE materials deparaffinization was done prior to isolation of DNA (Banerjee et al., 1995). For the isolation of cell-free DNA (cfDNA), 30 mL of peripheral blood was pooled and centrifuged for 10 min at 800 g within 24 h after the blood draw. Subsequently plasma was removed and snap frozen at −80 °C. Cell-free DNA was isolated from 3 × 1 mL plasma and eluted in 20 μL buffer using the QIAamp circulating nucleic acid kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Subsequently the eluate from 3 × 1 mL of plasma was used for each assay (IonPGM, dPCR and OnTarget) (Supplementary Table 1). DNA from whole blood samples was isolated using the QIAamp Blood Mini kit (Qiagen), according to the manufacturer's instructions. Extracted DNA was quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher, Waltham, MA, USA).

2.3. Targeted NGS using IonPGM

A CRC-specific 21-gene panel was established based on the top 19 most frequently mutated genes for CRC in the Catalogue of somatic mutations in cancer (COSMIC, June 2014). Based on the clinical relevance of *NRAS* in CRC (De Roock et al., 2010) and recent literature suggesting that *KDR* is a marker for hypermutation in CRC (Lee et al., 2014), these genes were also included in the panel. This resulted in a 21-gene panel consisting of *TP53*, *APC*, *KRAS*, *ATM*, *PTEN*, *PIK3CA*, *BRAF*, *FBXW7*, *SMAD4*, *NF1*, *RB1*, *ARID1A*, *PTCH1*, *CREBBP*, *KIT*, *KMT2D*, *CDH1*, *MLH1*, *EGFR*, *NRAS* & *KDR*. These 21 genes were included in a custom amplicon-based sequencing panel (1115 amplicons, ~89 kb), where the amplicons covered the whole exome of the gene, except for genes *KRAS*, *PIK3CA*, *BRAF*, *EGFR* and *NRAS* in which only the hotspot regions were sequenced (Supplemental Table 2). Libraries for these genes were constructed using the Ion AmpliSeq library kit 2.0 (Thermo Fisher) and subsequently the libraries were sequenced on the Ion Torrent NGS platform (Thermo Fisher), all as described before (Weerts et al., 2016).

IonPGM data was analyzed using our previously described standard calling pipeline (Jansen et al., 2016; Weerts et al., 2016). In short, raw IonPGM data was first loaded into the TorrentSuite variant caller 4.3, and variants were called with somatic low stringency setting. Then additional filtering was applied: variants were excluded if they were not present in the targeted exonic regions, if they were present in ≥90% of the samples, if they had a Q-score of ≤20, strand bias of ≥90%, read depth ≤100 (≤20 for normal tissue) or mutant allele read depth of ≤10. Then variants detected in normal tissue with a variant allele frequency of ≥35% and/or variants present in the virtual normal database (Hiltemann et al., 2015) were excluded as somatic variants. Lastly, all detected variants were inspected in all patient-matched samples using raw data without any of the filtering steps (IonPGM hotspot file). The remaining variants were considered to be true somatic variants.

A modified approach to sensitively call known variants in cfDNA was also evaluated. In this modified calling pipeline, all variants observed in the primary tumor or the metastasis as identified with the standard calling pipeline were analyzed in the cfDNA. All of the above criteria to call a variant were used, except for the criterion that variants had to be called by the TorrentSuite variant caller. The somatic low stringency calling was omitted in this approach, as this calling was originally developed for variant calling in tissue, in which variant allele frequencies are usually higher than in cfDNA.

2.4. NGS preceded by OnTarget enrichment

DNA isolated from all plasma samples and a selected set of tissue samples and whole blood samples were sent to Boreal Genomics (Vancouver, BC, Canada), and processed with the OnTarget assay (Thompson et al., 2012) followed by targeted sequencing on the MiSeq platform (Illumina, San Diego, CA, USA) as described before (Kidess et al., 2015). Boreal Genomics was blinded from all results obtained with IonPGM and digital PCR. The OnTarget assay targets 96 mutations in 9 genes (*BRAF*, *CTNNB1*, *EGFR*, *KRAS*, *FOXL2*, *GNAS*, *NRAS*, *PIK3CA* and *TP53*; Supplementary Table 3). The assay detects mutant DNA by electrophoretically removing wild-type DNA from the sample before significant PCR amplification or sequencing. Mutations were called positive if they were detected above a limit of detection (LOD), which was calculated as the mean plus 3 standard deviations of the mutant background (as observed on >100 known wild-type samples), plus two copies of the mutant. In cases where a mutant was called positive on a given PCR amplicon, the LOD for all other mutants on the same amplicon was raised by 1% of the detected mutant abundance to prevent false positives from PCR errors on the detected mutant. The limit of detection was calculated separately for each mutation in each sample to maximize assay sensitivity and specificity. Further details regarding the OnTarget methods are described in the Supplementary Methods. To evaluate the linearity, accuracy, and precision of the OnTarget assay, a set of samples designed for validation of cfDNA assays (Multiplex I cfDNA Reference Standard, from Horizon DX, Cambridge UK) were tested. The validation experiments (its methods discussed in detail in the Supplementary Methods) demonstrated high sensitivity and specificity of the assay (Supplementary Table 4).

2.5. Digital PCR

Digital PCR for somatic variants was performed using validated Taqman SNP genotyping assays for *KRAS* p.G12D, *KRAS* p.G12V, *KRAS* p.G13D, *PIK3CA* p.E545K, *TP53* p.R273H and *TP53* p.R248Q (Thermo Fisher) on the Quantstudio 3D digital PCR system (Thermo Fisher), according to the manufacturer's instructions. In case of cfDNA, the maximum input of 7.8 μL DNA was used. The presence of at least 2 mutant signals (FAM-positive, VIC-negative) was considered positive for a certain mutation.

To assess the proportion of cfDNA fragments, a Taqman *β-actin* dPCR assay was used based on the assay described by Norton et al. (2013) which is able to detect small (≥136 bp) and long (≥420 bp) *β-actin* fragments. This assay was used to quantify the extent of leukocyte lysis, which is characterized by an increased number of large DNA fragments relative to small DNA fragments (Van Dessel et al., 2016). A standardized input of 2 ng was used for the assay, to minimize the chances of double-positive events related to high input. Double-positive events (positive for ≥136 bp & ≥420 bp) were classified as long fragments ≥2000 bp, as the primers for the 136 bp and 420 bp span about 2000 bp. All dPCR experiments were analyzed in the Quantstudio 3D AnalysisSuite (Thermo Fisher) by one experienced technician (JH).

2.6. Statistical considerations

Our primary endpoint was the feasibility of ionPGM sequencing on ctDNA using our standard algorithm. Secondary endpoints included to explore if ctDNA is more resembling of the primary tumor or the metastases, to explore associations between the number of circulating tumor cells and ctDNA detection, to explore associations between cfDNA fragmentation and ctDNA detection, and to explore how ionPGM sequencing relates to other methods detecting mutations in ctDNA. As this study was meant to be exploratory, no formal statistics were performed to compare groups or methods.

3. Results

3.1. Patients and tissues

Twelve patients were identified whom matched the inclusion criteria (Supplementary Table 5). Six patients presented with synchronous metastases, and 6 patients had metachronous metastases. Median number of days between the resection of the primary tumor and the resection of the liver metastases for the patients with metachronous metastases was 830 days (range 270–2522 days). None of the patients received adjuvant therapy after surgery of the primary tumor or induction-therapy prior to the surgery for the liver metastases. In the set of 36 tissue samples (primary tumor, metastases and normal liver or colon), 25 tissue samples were FF and 11 tissue samples were FFPE. The median tumor cell percentage was 80% (interquartile range 20%).

3.2. Plasma isolation and raw analysis of samples

Median cfDNA concentration after isolation was 564 pg/μL (range 442–1224). A total of 5.75–15.91 ng cfDNA was sequenced on the IonPGM platform (Supplementary Table 6). Median coverage was 751x (range 582x–1141x) for tissue samples and 728x (range 527x–812x) for cfDNA samples. Raw data was analyzed and variants not meeting our criteria for somatic mutations were excluded with the exception of one variant (TP53 p.R273H). This variant was observed in one of the 478 virtual normal genomes (0.2%), but since this variant is enriched in TCGA CRC data (3,3%) (Cancer Genome Atlas, 2012) and linked to enhanced proliferation and invasion (Li et al., 2014) it was evaluated as a somatic variant. Following further filtering of variants using the standard variant calling pipeline, a median of 3 variants were called per primary tumor or metastasis. However, in the primary tumors of patients 1, 4 & 5 more than 200 variants remained after filtering. Further investigation of these variants revealed that >97% of the called variants were C > T or G > A variant substitutions, which had previously been linked to sequencings artefacts caused by formalin fixation (Do and Dobrovic, 2012; Do et al., 2013). In an attempt to reduce these artefacts one FFPE-derived DNA was treated with uracil-DNA glycosylase (UDG), which has been described to reduce FFPE-related sequencing artefacts (Do and Dobrovic, 2012; Do et al., 2013). While a great reduction of variants was observed after UDG treatment, still more than 100 variants remained after filtering (data not shown). Since these FFPE-related artefacts severely limited somatic mutation detection, the primary tumor FFPE samples of patients 1, 4 & 5 were omitted from further analysis.

3.3. Concordance between primary tumor, metastasis and cfDNA using IonPGM NGS

Following standard filtering 28 variants were observed in the primary tumor and 33 variants in the metastases, comprising a total of 29 distinct variants (Table 1). Of these 29 variants, 10 variants were not previously described in COSMIC. Concordance of all variants between the primary tumor and the metastases was 72%. In the cfDNAs, a total of 11 variants were observed in the blood of 6 patients. Two variants were found exclusively in ctDNA (*KMT2D* p.G794R & *ATM* p.A2301T) which were not previously described in COSMIC. From 28 variants observed in the primary tumor, 5 were retrieved in cfDNA (18%). Of note is that 4 of these 5 retrieved variants were observed in the same patient (patient 9). Out of 33 variants observed in the metastases, 9 variants were retrieved in the cfDNA (27%).

3.4. Investigation of modified calling pipeline for cfDNA

To explore whether the sensitivity for calling variants in cfDNA from plasma with the standard filtering strategy could be improved, variants previously observed in the primary tumor or the metastasis were investigated for their presence in cfDNA using a modified calling pipeline. For this pipeline, the variant in cfDNA did not have to be called by the TorrentSuite variant calling program. The modified calling approach led to the identification of 9 additional variants in cfDNA

Table 1 – Variants called in the primary tumor, metastases and plasma.

	Patient	Gene	Position	COSMIC	Primary ionPGM	Meta ionPGM	Plasma ionPGM
Metachronous	1	APC	p.Q1388X	Yes	X	54	2.7
		APC	p.R858X	Yes	X	27.4	.
	2	CREBBP	p.P2383L	No	5.4	.	.
		FBXW7	p.D399Y	No	19.8	39.4	.
		KRAS	p.G12D	Yes	28.6	38.6	.
		PIK3CA	p.E545K	Yes	41.1	74.8	.
		TP53	p.G108S	Yes	40.9	78.4	.
		TP53	p.L130I	No	44.1	74.7	.
	3	APC	p.C1369X	No	52.0	78.2	3.7
		CREBBP	p.P2383S	No	6.0	.	.
		TP53	p.R273H	Yes	50.6	82.0	7.3
	4	APC	p.R223X	Yes	X	66.8	25.8
	5	APC	p.E1288X	Yes	X	5.4	.
		APC	p.R284X	Yes	X	10	1.6
		TP53	p.R248Q	Yes	X	17.9	2.2
	6	APC	p.R481X	Yes	.	76.2	15.8
		FBXW7	p.R689W	Yes	22.0	.	.
		FBXW7	p.S582L	Yes	23.8	.	.
		PIK3CA	p.E545K	Yes	5.3	.	.
		KRAS	p.G12V	Yes	.	49.7	5.3
		TP53	p.R273H	Yes	.	72.3	12.2
Synchronous	7	KRAS	p.G12D	Yes	12.7	8.5	.
		ATM	p.A2301T	No	.	.	3.4
	8	APC	p.E1390X	Yes	22.3	50.7	1.3
		KRAS	p.G12D	Yes	30.9	51.2	2.3
		TP53	p.R175H	Yes	37.2	69.2	.
		KMT2D	p.G794R	No	.	.	4.2
	9	APC	p.R858X	Yes	24.4	41.2	17.2
		CREBBP	p.P937Q	No	4.2	4.3	7.2
		KRAS	p.G12D	Yes	39.7	35.0	20.5
		TP53	p.R248Q	Yes	37.5	55.7	14.1
	10	TP53	p.R282W	Yes	66.8	61.7	.
	11	APC	p.R481X	Yes	23.9	16.3	2.9
		TP53	p.M237I	Yes	55.4	37.3	1.3
	12	APC	p.E1390X	Yes	26.0	20.9	.
		APC	p.E564X	No	19.1	12.5	.
		CREBBP	p.P975S	No	.	4.2	.
		CREBBP	p.R601Q	No	13.0	9.8	.
		KRAS	p.G13D	Yes	37.9	26.6	1.5
		PIK3CA	p.E545K	Yes	18.0	12.9	.

Variants in plasma only called after modified variant calling are in red. Excluded samples because of FFPE-related artefacts are indicated with an "X". Not detected variants are indicated with a dot.

and showed variants in the blood of 10 patients (Table 1). In the primary tumor now 11 of 28 variants could be retrieved in the cfDNA samples (39% compared to 18% with standard filtering). In the metastases 18 of 33 variants were retrieved in the cfDNA samples (55% compared to 27% with standard filtering).

After using the modified pipeline, 20 different variants in cfDNA (5 in cfDNA versus primary tumor, 3 in cfDNA versus metastases, 12 in cfDNA versus both primary tumor and metastases) still remained undetected. When the raw data for these variants was explored (Supplementary Table 7), for 10 variants no mutant reads were observed at all in cfDNA, while for 5 other variants the number of mutant reads did not exceed the number of mutant reads found in cfDNA of patients without that variant found in their tissue. For 5 additional variants the number of mutant reads did exceed the number of mutant reads observed in cfDNA of patients without that variant in tissue, however, these were generally low-confidence variants with Q-scores below 20.

3.5. *OnTarget enrichment followed by NGS*

To explore whether enrichment of mutant alleles with the OnTarget technique followed by NGS would improve sensitivity compared to IonPGM NGS, all cfDNA samples and normal tissues were analyzed with the OnTarget assay (Supplementary Table 8). A total of 3.0–10.0 ng cfDNA was used for the procedure, which was a similar input as used with IonPGM. The OnTarget assay covers 96 hotspot mutations in 9 genes, and based on our IonPGM sequencing in the primary tumor and metastases, a maximum of 15 mutations could potentially be detected by the OnTarget assay in the samples of 9 patients included in our study (for 3 patients the OnTarget assay did not comprise the observed mutations

in the tissues of these patients). These 15 mutations were detected with IonPGM in 5 cfDNA samples with the standard pipeline (33%) and in 8 cfDNA samples with the modified pipeline (58%). Using the OnTarget assay 12 out of 15 variants (80%) could be retrieved in the cfDNA (Table 2). No additional variants were detected in cfDNA with the OnTarget assay. Interestingly, the OnTarget assay also detected a total of 13 variants in 7 of 9 normal tumor-adjacent tissues (Supplementary Table 8). For 5 patients in which mutations in tumor-adjacent normal tissue were observed there was whole blood available, and all of these variants were absent in whole blood.

3.6. Digital PCR

For 14 mutations observed in the primary tumor and/or the metastases, we had validated dPCR assays available (Supplementary Table 9). Using dPCR, 13 of 14 mutations (93%) observed in the primary tumor and/or the metastases were detected (Table 2). Of note is that the one mutation that was not detected was only observed in the primary tumor of patient 6 at a low frequency (5%), and not the metastases. Digital PCR was however able to detect all mutations that occurred in the liver metastasis of this particular patient. Compared to the OnTarget assay, dPCR detected two additional mutations, both in patient 2. Again, a significant number of mutations (9 out of 14) were detected in tumor-adjacent tissue with normal appearing histology (Supplementary Table 10), confirming the findings with the OnTarget assay. None of these mutations were observed in whole blood.

3.7. CTC enumeration & DNA fragmentation assay

To gain more insight into why ctDNA was or was not detected in some samples, the number of CTCs and fragmentation of cfDNA were assessed. CTC enumeration results of the main study were retrieved (Lalmahomed et al., 2015) for each patient at the time of cfDNA isolation (Table 3). In 4 patients, no CTCs were detected in 30 mL of blood, and in 1 of these patients, mutations in ctDNA were detected using IonPGM with our standard calling pipeline. The use of the modified pipeline, the OnTarget assay or dPCR however led to the identification of mutations in most patients, including the patients without CTCs. Because EDTA blood was used that was processed within 24 h, it was also evaluated whether large DNA fragments from lysed leukocytes diluted out small DNA fragments and decreased sensitivity for ctDNA analyses. A dPCR-based assay was used to detect small fragments (136 bp) and large fragments (>400 bp), the latter indicative of the presence of large DNA fragments from lysed leukocytes. The median percentage of small DNA fragments out of the total number of fragments (small + large) was 65% (range 46–73%; Table 3). When patients were separated in two groups using the median of small fragments, we found that mutations in ctDNA using IonPGM and modified filtering were detected in 5 of 6 patients (in which 53% of all potentially detectable mutations were detectable) with a number of small fragments above the median, and in 5 of 6 patients (in which 62% of all potentially detectable mutations were detectable) with a number of small fragments below the median.

4. Discussion

In the era of precision medicine in oncology, there is a high need for accurate biomarkers that can be used before, during and after treatment in a minimally invasive way. Assessing ctDNA has sparked much interest to become such a biomarker. To date, most reports using ctDNA in CRC have reported on strategies using a limited set of genes (most often *KRAS*, *BRAF* & *PIK3CA*) (Bettegowda et al., 2014; El Messaoudi et al., 2016; Kidess et al., 2015; Mouliere et al., 2013; Siravegna et al., 2015; Tabernero et al., 2015; Thierry et al., 2014) or an approach in which personalized assays based on mutations found in the primary tumor or metastases were

Table 2 – Detection of plasma ctDNA mutations using various techniques and strategies.

Patient	Gene	Position	Plasma IonPGM standard filtering VAF%	Plasma ionPGM modified filtering VAF%	Plasma OnTarget VAF%	Plasma dPCR VAF%
2	KRAS	p.G12D	.	.	.	1.94%
	PIK3CA	p.E545K	.	.	.	2.23%
3	TP53	p.R273H	7.3%	7.3%	3.5%	7.03%
5	TP53	p.R248Q	.	2.2%	1.5%	2.7%
6	KRAS	p.G12V	5.3%	5.3%	3.6%	8.5%
	TP53	p.R273H	12.2%	12.2%	4.9%	7.03%
	PIK3CA	p.E545K	.	.	.	.
7	KRAS	p.G12D	.	.	0.92%	1.57%
8	KRAS	p.G12D	.	2.3%	1.6%	1.66%
	TP53	p.R175H	.	.	2.8%	No assay
9	KRAS	p.G12D	20.5%	20.5%	14.0%	12.87%
	TP53	p.R248Q	14.1%	14.1%	20.0%	15.02%
10	TP53	p.R282W	.	.	0.67%	2.94%
12	KRAS	p.G13D	.	1.5%	3.1%	3.96%
	PIK3CA	p.E545K	.	.	2.7%	2.9%

Not detected variants are indicated with a dot. VAF% = variant allele frequency.

Table 3 – Associations between the number of CTCs, the percentage of small fragments and detection of somatic mutations with ionPGM using two filtering variants, and with OnTarget and dPCR.

Patient	Number of CTCs/30 mL	% of small DNA fragments	Mutations present in plasma with standard filtering	Mutations present with modified filtering	Mutations present with OnTarget assay	Mutations present with digital PCR
9	0	46%	Yes (4 of 4)	Yes (4 of 4)	Yes (2 of 2)	Yes (2 of 2)
10	0	55%	No	No	Yes (1 of 1)	Yes (1 of 1)
11	0	64%	No	Yes (2 of 2)	Not applicable	Not applicable
1	0	70%	No	Yes (1 of 2)	Not applicable	Not applicable
7	1	61%	Yes (2 of 3)	Yes (2 of 3)	Yes (1 of 1)	Yes (1 of 1)
12	1	63%	No	Yes (1 of 6)	Yes (2 of 2)	Yes (2 of 2)
3	1	66%	Yes (2 of 3)	Yes (2 of 3)	Yes (1 of 1)	Yes (1 of 1)
4	1	72%	Yes (1 of 1)	Yes (1 of 1)	Not applicable	Not applicable
8	2	62%	Yes (2 of 5)	Yes (4 of 5)	Yes (2 of 2)	Yes (2 of 2)
2	5	69%	No	No	No	Yes (2 of 2)
5	8	73%	No	Yes (2 of 3)	Yes (1 of 1)	Yes (1 of 1)
6	35	71%	Yes (3 of 6)	Yes (3 of 6)	Yes (2 of 3)	Yes (2 of 3)

For OnTarget and dPCR, only mutations previously found in the primary tumor or metastases are reported in this table.
Shadings represent the extent of concordance between tissue and plasma. "Not applicable" means that the mutation was not included in the panel (OnTarget) or that there was no available assay (dPCR) for the mutation as observed with ionPGM sequencing.

developed to detect ctDNA (Reinert et al., 2015; Tie et al., 2015). Of note is that this latter approach also focuses on a limited set of somatic mutations only. The current study reported on the feasibility of a NGS panel on ctDNA in mCRC patients covering the 21 most prevalent and relevant genes in CRC known to date. It was observed that NGS with IonPGM is feasible on cfDNA, however only a limited number of variants observed in the primary tumor and the metastases could be retrieved in the cfDNA. A number of specific variants not observed in cfDNA with IonPGM could however be detected using alternative methods such as OnTarget enrichment followed by NGS and dPCR.

The sensitivity of IonPGM sequencing with standard filtering to retrieve mutations found in tissue in ctDNA was less than expected. As the plasma was not optimally collected (out of EDTA blood within 24 h of the blood draw), the percentage of small fragments in cfDNA as a measure of leukocyte lysis was assessed. In addition, the number of CTCs was assessed as the number of CTCs was previously described to be associated with the probability to detect ctDNA in breast cancer (Dawson et al., 2013). However, both the extent of leukocyte lysis as well as the number of CTCs did not provide obvious explanations as to why mutant ctDNA was or was not detected in some patients, although the power of this analysis was limited due to the small sample size of the presented cohort. Interestingly, for example, is patient 9, in whom ctDNA variant allele frequencies of 7–20% were observed, but in whom no CTCs were detected and who had the lowest percentage of small DNA fragments. This illustrates that we have limited insight into why some patients have high or low ctDNA frequencies. The ratio of ctDNA versus wildtype cfDNA probably plays an important role, however to date our understanding of this ratio, or a measure how to quantify it, is lacking.

The observation that a modified calling pipeline for ctDNA, instead of a standard calling pipeline based on calls by the TorrentSuite variant caller, resulted in an increased detection of mutations as found in tissue is in accordance with Couraud et al. (2014). This group performed IonPGM sequencing on ctDNA in patients with non-small cell lung cancer and observed an increase in concordance rate between matched tissue and ctDNA from 16% to 58% using an in-house calling algorithm instead of using standard IonPGM variant calling. Also similar to our results with the modified calling pipeline is the report by Frenel et al. (2015) whom reported that 59% of variants observed in metastases of patients with various tumors could be retrieved with IonPGM sequencing. However, others have also reported higher concordance between tissue and ctDNA using IonPGM of >80–90% (Lebofsky et al., 2014; Rothe et al., 2014). A reason for the lower concordance as observed in our study may be that in our study CRC patients with oligometastatic disease were included, while in the studies with higher concordances heavily pretreated patients were included with probably a higher tumor load. This is also reflected by the fact that in these studies, very high ctDNA variant frequencies of >50% were observed in multiple patients.

The presented results of high concordance between tissue and ctDNA when applying dPCR is in accordance with previous reports using similar techniques (Bettegowda et al., 2014; Mouliere et al., 2013; Reinert et al., 2015; Tabernero et al., 2015; Thierry et al., 2014; Tie et al., 2015). High concordance between tissue and ctDNA with OnTarget followed by NGS was also observed in the current study, which is consistent with a previous report on this assay (Kidess et al., 2015). Interestingly, using dPCR and the OnTarget assay somatic mutations were observed at very low frequencies in the tumor-adjacent control tissue with normal-appearing histology in

the majority of patients, which were also observed in the matched tissue samples. As the majority of these mutations were observed by both methods, and the fact that none of these mutations were detected in whole blood of the same patient, we believe these are true somatic mutations present in normal-appearing tumor-adjacent tissue. An explanation for this finding may be that tumor DNA or tumor cells diffused or migrated into the surrounding normal tissue. Further examination of these findings is warranted, for example by investigating whether mutations in normal-appearing tissue are related to an increased chance of local disease relapse.

The study as presented here has some limitations. The small sample size, combined with having to omit 3 primary tumor samples from the analysis due to FFPE-related variant noise, together with the low sensitivity of IonPGM sequencing to detect mutations, makes exploration whether or not ctDNA resembles the metastases rather than the primary tumor impossible. Also for this reason differences between patients with metachronous metastases versus synchronous metastases could not be explored. This data does however provide perspectives on the advantages and disadvantages of current ctDNA methods for detecting somatic mutations, and which one to use for which particular research purpose.

At least in this group of patients with oligometastatic disease, IonPGM sequencing on ctDNA lacks sensitivity to detect mutations, especially if the tissue mutational status is unknown. If the tissue mutational status is known, IonPGM with the modified calling pipeline is a feasible option but still sensitivity remains an issue to evaluate mutations in multiple genes in ctDNA. However, NGS of multiple genes like we performed here is probably the only option if resistance mutations not present in tissue and not previously reported need to be identified. This is also reflected by the fact that two novel mutations in ctDNA were identified that have not been previously reported. However, given the limited sensitivity of IonPGM sequencing to detect mutations this approach is likely to be of restricted value since many variants remain undetected. IonPGM sequencing of ctDNA in patients with higher tumor loads may however yield better results.

If one would like to monitor certain mutations during treatment, an NGS approach using the primary tumor or metastases for discovery and subsequent tracing of mutations with dPCR is likely a successful approach, given the high sensitivity to detect mutations with dPCR as described here. Reinert et al. (2015) reported on this approach and developed 2 to 6 individualized dPCR assays per patient for a total of 11 patients. They observed 100% sensitivity and 100% specificity to detect recurrence of CRC using these individualized dPCRs. However, disadvantages of such an approach are labor-intensity because many individualized dPCR assays have to be produced. In addition, novel or known mutations causing resistance not present in the tissue, or developing during treatment, are missed. Alternatively, the OnTarget assay had high sensitivity to detect many hotspot mutations, tackling the issue of labor-intensity and potentially of mutations not present in the tumor tissue. While dPCR seemed to be slightly more sensitive than the OnTarget assay to detect mutations in ctDNA and tumor-adjacent tissue, the OnTarget assay identified some mutations in tumor-adjacent normal tissue that were not detected in the matched tumor tissue. These mutations would certainly have been missed by an approach using dPCR assays based on previous findings in tumor tissue. Nonetheless, the OnTarget assay is still bound to a limited repertoire of mutations, and currently unknown resistance mutations are potentially missed by the technique. Finally, our sample size is limited to make firm conclusions about the OnTarget assay versus dPCR. At the very least, the OnTarget assay seems like an attractive option for ctDNA detection to screen for multiple mutations if no tumor tissue is available.

In summary, three targeted methods to detect somatic mutations in ctDNA were described and pros and cons were provided for each method. Future efforts using ctDNA as a tool to detect somatic mutations in cancer patients should carefully consider all available methods for ctDNA detection and choose the method most fit to answer the specific research question.

Conflicts of interest

M. Wiggin is a shareholder and employee of Boreal Genomics. A. Marziali is a shareholder, director, and employee of Boreal Genomics.

Grant support

This work was partly supported by a grant from the Cancer Genomics Netherlands (CGC.nl)/Netherlands Organization for Scientific Research (NWO), and a grant from the Dutch Cancer Society (KWF; grant number EMCR 2014-6340). M. Weerts received research support from Philips Research.

Acknowledgements

The authors would like to thank Dirk Grunhagen for the collection of tissue materials. In addition, we thank Ronald van Marion & Winand Dinjens of the department of Pathology for facilitating the IonPGM sequencing.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.10.001.

REFERENCES

Banerjee, S.K., Makdisi, W.F., Weston, A.P., Mitchell, S.M., Campbell, D.R., 1995. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. Biotechniques 18, 768–770, 772–763.

Bettegowda, C., Sausen, M., Leary, R.J., Kinde, I., Wang, Y., Agrawal, N., Bartlett, B.R., Wang, H., Luber, B., Alani, R.M., Antonarakis, E.S., Azad, N.S., Bardelli, A., Brem, H.,

Cameron, J.L., Lee, C.C., Fecher, L.A., Gallia, G.L., Gibbs, P., Le, D., Giuntoli, R.L., Goggins, M., Hogarty, M.D., Holdhoff, M., Hong, S.M., Jiao, Y., Juhl, H.H., Kim, J.J., Siravegna, G., Laheru, D.A., Lauricella, C., Lim, M., Lipson, E.J., Marie, S.K., Netto, G.J., Oliner, K.S., Olivi, A., Olsson, L., Riggins, G.J., Sartore-Bianchi, A., Schmidt, K., Shih, I.M., Oba-Shinjo, S.M., Siena, S., Theodorescu, D., Tie, J., Harkins, T.T., Veronese, S., Wang, T.L., Weingart, J.D., Wolfgang, C.L., Wood, L.D., Xing, D., Hruban, R.H., Wu, J., Allen, P.J., Schmidt, C.M., Choti, M.A., Velculescu, V.E., Kinzler, K.W., Vogelstein, B., Papadopoulos, N., Diaz Jr., L.A., 2014. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci. Transl. Med. 6, 224ra224.

Cancer Genome Atlas, N., 2012. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337.

Couraud, S., Vaca-Paniagua, F., Villar, S., Oliver, J., Schuster, T., Blanche, H., Girard, N., Tredaniel, J., Guilleminault, L., Gervais, R., Prim, N., Vincent, M., Margery, J., Larive, S., Foucher, P., Duvert, B., Vallee, M., Le Calvez-Kelm, F., McKay, J., Missy, P., Morin, F., Zalcman, G., Olivier, M., Souquet, P.J.Bio, C.I.i, 2014. Noninvasive diagnosis of actionable mutations by deep sequencing of circulating free DNA in lung cancer from never-smokers: a proof-of-concept study from BioCAST/IFCT-1002. Clin. Cancer Res. 20, 4613–4624.

Dawson, S.J., Rosenfeld, N., Caldas, C., 2013. Circulating tumor DNA to monitor metastatic breast cancer. N. Engl. J. Med. 369, 93–94.

De Roock, W., Claes, B., Bernasconi, D., De Schutter, J., Biesmans, B., Fountzilas, G., Kalogeras, K.T., Kotoula, V., Papamichael, D., Laurent-Puig, P., Penault-Llorca, F., Rougier, P., Vincenzi, B., Santini, D., Tonini, G., Cappuzzo, F., Frattini, M., Molinari, F., Saletti, P., De Dosso, S., Martini, M., Bardelli, A., Siena, S., Sartore-Bianchi, A., Tabernero, J., Macarulla, T., Di Fiore, F., Gangloff, A.O., Ciardiello, F., Pfeiffer, P., Qvortrup, C., Hansen, T.P., Van Cutsem, E., Piessevaux, H., Lambrechts, D., Delorenzi, M., Tejpar, S., 2010. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 11, 753–762.

Diaz Jr., L.A., Bardelli, A., 2014. Liquid biopsies: genotyping circulating tumor DNA. J. Clin. Oncol. 32, 579–586.

Diehl, F., Li, M., He, Y., Kinzler, K.W., Vogelstein, B., Dressman, D., 2006. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat. Methods 3, 551–559.

Diehl, F., Schmidt, K., Choti, M.A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S.A., Kinzler, K.W., Vogelstein, B., Diaz Jr., L.A., 2008. Circulating mutant DNA to assess tumor dynamics. Nat. Med. 14, 985–990.

Do, H., Dobrovic, A., 2012. Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil- DNA glycosylase. Oncotarget 3, 546–558.

Do, H., Wong, S.Q., Li, J., Dobrovic, A., 2013. Reducing sequence artifacts in amplicon-based massively parallel sequencing of formalin-fixed paraffin-embedded DNA by enzymatic depletion of uracil-containing templates. Clin. Chem. 59, 1376–1383.

El Messaoudi, S., Mouliere, F., Du Manoir, S., Bascoul-Mollevi, C., Gillet, B., Nouaille, M., Fiess, C., Crapez, E., Bibeau, F., Theillet, C.G., Mazard, T., Pezet, D., Mathonnet, M., Ychou, M., Thierry, A.R., 2016. Circulating DNA as a strong multi-marker prognostic tool for metastatic colorectal cancer patient management care. Clin. Cancer Res. 22, 3067–3077.

Frenel, J.S., Carreira, S., Goodall, J., Roda, D., Perez-Lopez, R., Tunariu, N., Riisnaes, R., Miranda, S., Figueiredo, I., Nava-Rodrigues, D., Smith, A., Leux, C., Garcia-Murillas, I., Ferraldeschi, R., Lorente, D., Mateo, J., Ong, M., Yap, T.A., Banerji, U., Gasi Tandefelt, D., Turner, N., Attard, G., de Bono, J.S., 2015. Serial next-generation sequencing of circulating cell-free DNA evaluating tumor clone response to molecularly targeted drug administration. Clin. Cancer Res. 21, 4586–4596.

Gerlinger, M., Rowan, A.J., Horswell, S., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., Varela, I., Phillimore, B., Begum, S., McDonald, N.Q., Butler, A., Jones, D., Raine, K., Latimer, C., Santos, C.R., Nohadani, M., Eklund, A.C., Spencer-Dene, B., Clark, G., Pickering, L., Stamp, G., Gore, M., Szallasi, Z., Downward, J., Futreal, P.A., Swanton, C., 2012. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N. Engl. J. Med. 366, 883–892.

Haber, D.A., Gray, N.S., Baselga, J., 2011. The evolving war on cancer. Cell 145, 19–24.

Haber, D.A., Velculescu, V.E., 2014. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov. 4, 650–661.

Hiltemann, S., Jenster, G., Trapman, J., van der Spek, P., Stubbs, A., 2015. Discriminating somatic and germline mutations in tumor DNA samples without matching normals. Genome Res. 25, 1382–1390.

Jansen, M.P., Martens, J.W., Helmijr, J., Beaufort, C.M., Van Marion, R., Krol, N.M.G., Monkhorst, K., Trapman-Jansen, A.M.A.C., Meijer-van Gelder, M.E., Weerts, M.J.A., Ramirez-Ardila, D., Dubbink, H.J., Foekens, J., Sleijfer, S., Berns, E.M., 2016. Cell-free DNA mutations as biomarkers in breast cancer patients receiving tamoxifen. Oncotarget (in press).

Kidess, E., Heirich, K., Wiggin, M., Vysotskaia, V., Visser, B.C., Marziali, A., Wiedenmann, B., Norton, J.A., Lee, M., Jeffrey, S.S., Poultsides, G.A., 2015. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget 6, 2549–2561.

Lalmahomed, Z.S., Mostert, B., Onstenk, W., Kraan, J., Ayez, N., Gratama, J.W., Grunhagen, D., Verhoef, C., Sleijfer, S., 2015. Prognostic value of circulating tumour cells for early recurrence after resection of colorectal liver metastases. Br. J. Cancer 112, 556–561.

Lebofsky, R., Decraene, C., Bernard, V., Kamal, M., Blin, A., Leroy, Q., Rio Frio, T., Pierron, G., Callens, C., Bieche, I., Saliou, A., Madic, J., Rouleau, E., Bidard, F.C., Lantz, O., Stern, M.H., Le Tourneau, C., Pierga, J.Y., 2014. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol. Oncol. 9, 783–790.

Lee, S.Y., Haq, F., Kim, D., Jun, C., Jo, H.J., Ahn, S.M., Lee, W.S., 2014. Comparative genomic analysis of primary and synchronous metastatic colorectal cancers. PLoS One 9, e90459.

Leto, S.M., Trusolino, L., 2014. Primary and acquired resistance to EGFR-targeted therapies in colorectal cancer: impact on future treatment strategies. J. Mol. Med. (Berl) 92, 709–722.

Li, J., Yang, L., Gaur, S., Zhang, K., Wu, X., Yuan, Y.C., Li, H., Hu, S., Weng, Y., Yen, Y., 2014. Mutants TP53 p.R273H and p.R273C but not p.R273G enhance cancer cell malignancy. Hum. Mutat. 35, 575–584.

Marziali, A., Pel, J., Bizzotto, D., Whitehead, L.A., 2005. Novel electrophoresis mechanism based on synchronous alternating drag perturbation. Electrophoresis 26, 82–90.

Misale, S., Yaeger, R., Hobor, S., Scala, E., Janakiraman, M., Liska, D., Valtorta, E., Schiavo, R., Buscarino, M., Siravegna, G., Bencardino, K., Cercek, A., Chen, C.T., Veronese, S., Zanon, C., Sartore-Bianchi, A., Gambacorta, M., Gallicchio, M., Vakiani, E., Boscaro, V., Medico, E., Weiser, M., Siena, S., Di Nicolantonio, F., Solit, D., Bardelli, A., 2012. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 486, 532–536.

Mostert, B., Sieuwerts, A.M., Bolt-de Vries, J., Kraan, J., Lalmahomed, Z., van Galen, A., van der Spoel, P., de Weerd, V., Ramirez-Moreno, R., Smid, M., Verhoef, C., IJzermans, J.N.M., Gratama, J.W., Sleijfer, S., Foekens, J.A., Martens, J.W.M., 2015. mRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. Mol. Oncol. 9, 920–932.

Mouliere, F., El Messaoudi, S., Gongora, C., Guedj, A.S., Robert, B., Del Rio, M., Molina, F., Lamy, P.J., Lopez-Crapez, E., Mathonnet, M., Ychou, M., Pezet, D., Thierry, A.R., 2013. Circulating cell-free DNA from colorectal cancer patients may reveal high KRAS or BRAF mutation load. Transl Oncol. 6, 319–328.

Norton, S.E., Lechner, J.M., Williams, T., Fernando, M.R., 2013. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. Clin. Biochem. 46, 1561–1565.

Reinert, T., Scholer, L.V., Thomsen, R., Tobiasen, H., Vang, S., Nordentoft, I., Lamy, P., Kannerup, A.S., Mortensen, F.V., Stribolt, K., Hamilton-Dutoit, S., Nielsen, H.J., Laurberg, S., Pallisgaard, N., Pedersen, J.S., Orntoft, T.F., Andersen, C.L., 2015. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. Gut 65, 625–634.

Rothe, F., Laes, J.F., Lambrechts, D., Smeets, D., Vincent, D., Maetens, M., Fumagalli, D., Michiels, S., Drisis, S., Moerman, C., Detiffe, J.P., Larsimont, D., Awada, A., Piccart, M., Sotiriou, C., Ignatiadis, M., 2014. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann. Oncol. 25, 1959–1965.

Siravegna, G., Mussolin, B., Buscarino, M., Corti, G., Cassingena, A., Crisafulli, G., Ponzetti, A., Cremolini, C., Amatu, A., Lauricella, C., Lamba, S., Hobor, S., Avallone, A., Valtorta, E., Rospo, G., Medico, E., Motta, V., Antoniotti, C., Tatangelo, F., Bellosillo, B., Veronese, S., Budillon, A., Montagut, C., Racca, P., Marsoni, S., Falcone, A., Corcoran, R.B., Di Nicolantonio, F., Loupakis, F., Siena, S., Sartore-Bianchi, A., Bardelli, A., 2015. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat. Med. 21, 827.

Tabernero, J., Lenz, H.J., Siena, S., Sobrero, A., Falcone, A., Ychou, M., Humblet, Y., Bouche, O., Mineur, L., Barone, C., Adenis, A., Yoshino, T., Goldberg, R.M., Sargent, D.J., Wagner, A., Laurent, D., Teufel, M., Jeffers, M., Grothey, A., Van Cutsem, E., 2015. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: a retrospective, exploratory analysis of the CORRECT trial. Lancet Oncol. 16, 937–948.

Thierry, A.R., Mouliere, F., El Messaoudi, S., Mollevi, C., Lopez-Crapez, E., Rolet, F., Gillet, B., Gongora, C., Dechelotte, P., Robert, B., Del Rio, M., Lamy, P.J., Bibeau, F., Nouaille, M., Loriot, V., Jarrousse, A.S., Molina, F., Mathonnet, M., Pezet, D., Ychou, M., 2014. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat. Med. 20, 430–435.

Thompson, J.D., Shibahara, G., Rajan, S., Pel, J., Marziali, A., 2012. Winnowing DNA for rare sequences: highly specific sequence and methylation based enrichment. PloS one 7, e31597.

Tie, J., Kinde, I., Wang, Y., Wong, H.L., Roebert, J., Christie, M., Tacey, M., Wong, R., Singh, M., Karapetis, C.S., Desai, J., Tran, B., Strausberg, R.L., Diaz Jr., L.A., Papadopoulos, N., Kinzler, K.W., Vogelstein, B., Gibbs, P., 2015. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann. Oncol. 26, 1715–1722.

Turner, N.C., Reis-Filho, J.S., 2012. Genetic heterogeneity and cancer drug resistance. Lancet Oncol. 13, e178–185.

Van Dessel, L.F., Beije, N., Helmijr, J., Vitale, S., Kraan, J., Look, M.P., De Wit, R., Sleijfer, S., Jansen, M.P., Martens, J.W., Lolkema, M.P., 2016. Application of Circulating Tumor DNA in Prospective Clinical Oncology Trials: Standardization of Pre-analytical Conditions (Submitted for publication).

Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz Jr., L.A., Kinzler, K.W., 2013. Cancer genome landscapes. Science 339, 1546–1558.

Wang, J., Ramakrishnan, R., Tang, Z., Fan, W., Kluge, A., Dowlati, A., Jones, R.C., Ma, P.C., 2010. Quantifying EGFR alterations in the lung cancer genome with nanofluidic digital PCR arrays. Clin. Chem. 56, 623–632.

Weerts, M.J.A., van Marion, R., Helmijr, J., Beaufort, C.M., Krol, N.M.G., Trapman, A.M.A.C., Foekens, J.A., Dinjens, W.N., Sleijfer, S., Jansen, M.P.H.M., Martens, J.W., Berns, E.M., 2016. Somatic Mutations Detected by Targeted NGS in Minute Amounts Serum Derived cfDNA (Submitted for publication).