Analysis of circulating cell free DNA (ccfDNA): A promising tool for personalized medicine and cancer therapy

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Background

Although significant progress has been made in the development of new therapy approaches, cancer remains one of the leading causes of death worldwide. In most cases cancer remains undetected until its advanced stages because up to now efficient screening techniques for early detection are not still available.
However recently published data indicate that circulating cell-free DNA (ccfDNA) could become a promising biomarker in cancer diagnosis, therapy and prognosis. The use of ccfDNA presents several conceptual advantages compared to classic genetic analysis via tumor-tissue sampling. CcfDNA analysis is non-invasive and enables day-to-day patient follow-up and monitoring of treatment response. Analysis of ccfDNA also allows detection of genetic and epigenetic alterations within the tumor. Careful analysis of these alterations could provide valuable information to tailor the clinician's choice of treatment.
To check the feasibility of this approach we started a pilot study with patients suffering from colorectal cancer in order to establish analysis of ccfDNA in a routine laboratory. Optimization and normalization of the Workflow of the pilot study covers all aspects of the complete procedure: starting with blood sampling, isolation of the ccfDNA, determination of its concentration and determination of tumor-derived ccfDNA part and its fragmentation.

Work flow

Prior to analysis tumor derived DNA-fragments are enriched by cold-PCR and presence of sequence variants are either shown by next generation sequencing (NGS) or - if the mutation is known - by quantitative PCR, digital PCR and by NGS. To validate results DNA is isolated from respective tumor specimen and genes which are known to be frequently mutated in colon Cancer are sequenced by use of appropriate gene panels on Ion Torrent Personal Genome Machine (PJM) or Ion Proton.

Methods

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissues
DNA was isolated manually from one or two micro dissected 5-µm-thick sections of the respective specimen according to manufacturers' protocols (QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany).

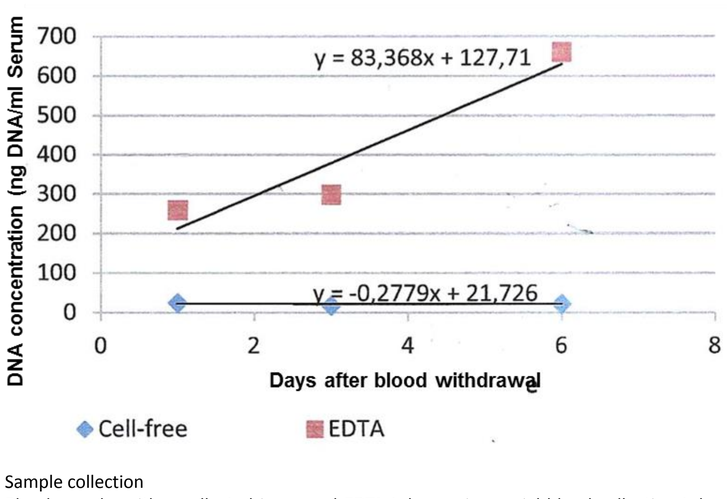
Plasma purification and ccfDNA extraction and sizing
Whole blood (9 ml) was drawn in a cell-free DNA blood collection tube (Streck, Omaha, NE USA). Plasma preparation was performed according to standard protocols and stored at -80° C. DNA was isolated from plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at −20 °C. DNA concentration was determined (Qubit 2.0, LifeTechnologies) and size distribution of plasma DNA fragments was evaluated (Agilent Technologies, Santa Clara, USA).

Gene Panels
A specific custom designed multiplex PCR panel was constructed using the AmpliSeq Designer (LifeTechnologies). This panel covers 97% of NRAS, PIK3CA, FBXW7, APC, BRAF, KRAS, TP53, SMAD4 with 212 PCR fragments amplified simultaneously in two PCR pools.

Ion Torrent PGM library preparation and DNA sequencing
Ion Torrent adapter-ligated library was constructed with the Ion AmpliSeq Library Kit 2.0 (Life Technologies). The resulting library was purified with AMPure beads (Beckman Coulter), and the library concentration and size was determined (Qubit 2.0, LifeTechnologies). Sample emulsion PCR, emulsion breaking, and enrichment were performed with the Ion PGM 200 Xpress Template Kit (Life Technologies). 316-chips were used to sequence barcoded samples on the Ion Torrent PGM for 65 cycles, and an Ion PGM 200 Sequencing Kit (Life Technologies) was used for sequencing reactions.

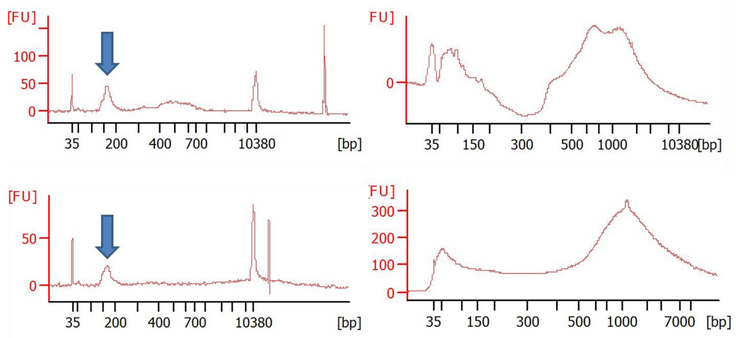
Variant calling
Initial data from the PGM runs were processed with the Ion Torrent platform-specific pipeline software Torrent Suite to generate sequence reads, then trim adapter sequences, filter, and remove poor signal-profile reads. In order to eliminate erroneous base calling and generate final variant calling, several filtering steps were used: defining average total coverage depth, variant coverage, variant frequency of each sample, and P value <0.01; visually inspecting and removing DNA strand-specific errors; defining variants within hotspots

Quality and stability in serum and/or tumors



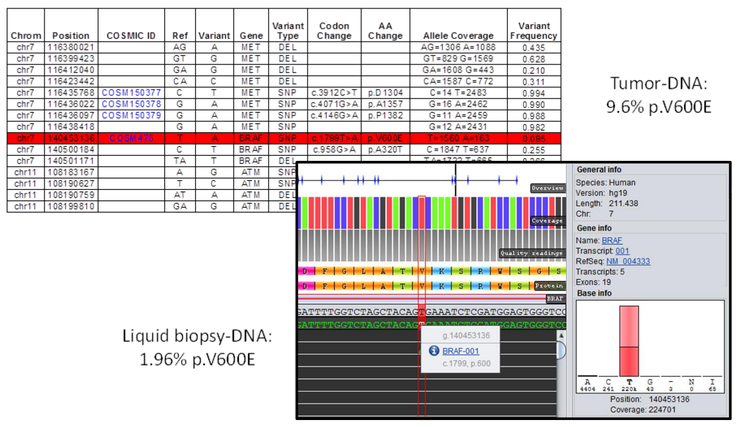
Sample collection
Blood samples either collected in normal EDTA tubes or in special blood collection tubes for stabilization of cell-free plasma DNA (Cell-Free DNA BCT®) were analyzed after 1, 3 and 6 days. DNA concentration/ml plasma was determined. DNA concentration in EDTA tubes increased according to storage time whereas DNA concentration in cell-free tubes remain constant indicating that blood cell lysis was detained and the amount of ccfDNA was not diluted by genomic DNA from lytic blood cells.

DNA-isolated from liquid biopsies or tumors is heavily fragmented



Size distribution of isolated plasma or tumor DNA was determined using the Agilent 2100 Bioanalyzer System. Data show that the size of DNA-fragments isolated from sera is well below 200 bp requiring special enrichment techniques.

Tumors are heterogeneous

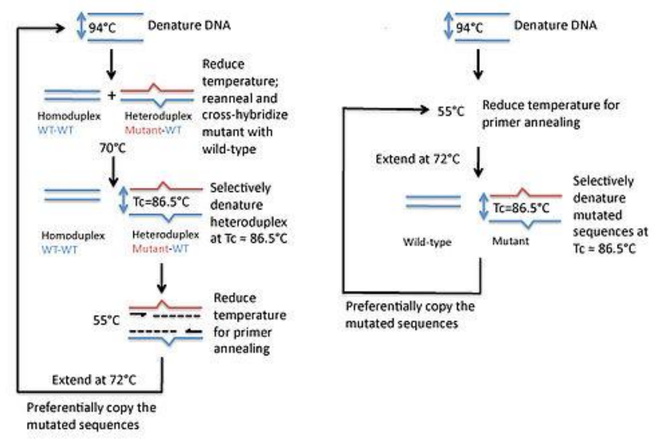


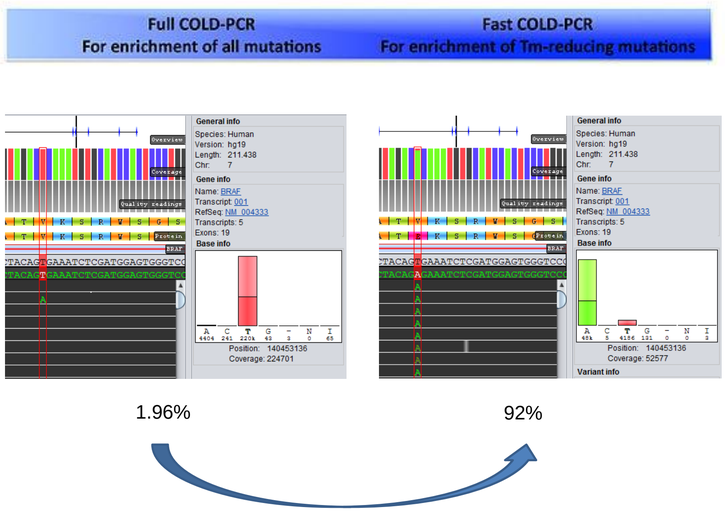
Tumor-DNA: 9.6% p.V600E

Liquid biopsy-DNA: 1.96% p.V600E

Tumor heterogeneity shown by low frequency of mutation (BRAF p.V600E) within isolated tumor DNA; this heterogeneity is present in the liquid biopsy too, however with much lower frequency. Low frequency of mutations hamper identification; therefore mutation-specific enrichment is necessary, e.g. by coldPCR

coldPCR enrichment of mutations



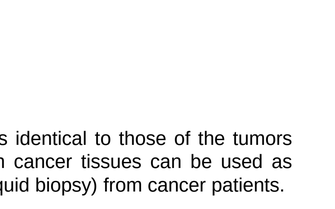


By „full cold PCR“ 50x enrichment of mutation was possible, which could be easily detected by conventional methods

No. Pat.	Sample	Stage	APC	KRAS	NRAS	BRAF	PIK3CA	TP53	FBXW 7	SMAD4
1	K1	IV	mutant	mutant						
2	K3	IV				mutant				
3	K5	IV		mutant	mutant					
4	K7	IV					mutant			
5	K22	IV	mutant	mutant						
6	K9	IV	mutant							mutant
7	K12	IIIB	mutant							
8	K17	IIIB		mutant						
9	K18	IIIB		mutant						
10	K20	IIIA					mutant			
11	K23	IIIB						mutant		
12	K2	IIIA			mutant					
13	K8	IIIA							mutant	
14	K14	IIA	mutant							
15	K16	IIA	mutant	mutant						
16	K25	IIA	mutant							
17	K11	I	mutant							
18	K15	I	mutant	mutant						
19	K19	I	mutant	mutant						
20	K24	I		mutant				mutant		
21	K26	I					mutant			
22	K13	0								

Sequence analysis of primary tumor material (FFPE) was performed on Ion Torrent Personal Genome Machine (PJM) or Ion Proton. In all tumor samples analyzed at least one gene had acquired a sequence variant/mutation which could be used as a biomarker for further monitoring.

Pts. No.	Stage	Mutant Genes	% reads in tumor	LB1- %reads	LB2- % reads	LB3- % reads
K 1*°	IV	APC1	41	41	41	n.m.
		APC2	26	13,3	0,07	n.m.
		KRAS	25	24	0,05	n.m.
K3	IV	BRAF**	42	38	50	50
K5	IV	NRAS**	19	1,1	0,2	0
K7	IV	PIK3CA	9	n.a.	n.m.	n.m.
K22	IV	KRAS	22	30	n.m.	n.m.
		APC	10	n.a.	n.m.	n.m.
K9*	IV	APC	37	0,04	0,01	n.m.
		SMAD4	7	n.r.	0,01	n.m.
K12	IIIB	APC1	27	3,2	0	0
		APC2	28	5,4	0	0
		TP53	62	8,3	0	0
		KRAS	37	4,8	0	0
K17	IIIB	KRAS**	20	1,1	n.m.	n.m.
K18	IIIB	KRAS	18	n.r.		
K20	IIIA	APC	5	0	0	n.m.
K23*	IIIB	TP53	32	n.a.	n.a.	n.a.
		KRAS1**	23	3(2,3)	2(1,2)	2(0,9)
		KRAS2	12	n.a.	n.a.	n.a.
K2*	IIIA	NRAS	40	5	0	0
K8	IIIA	FBXW7	13	n.a.	n.a.	n.m.
K14	IIA	APC	36	n.r.		
K16	IIA	KRAS**	28	8(6,3)	27(22)	n.m.
		APC	41	10	10	n.m.
K25	IIA	APC	28	n.r.		
K11	I	APC	15	0	0	n.m.
K15	I	APC	9	0	0	n.m.
K19	I	KRAS**	36	0	0	n.m.
		APC	19	0	0	n.m.
K24	I	KRAS	23	n.r.		
K26	I	PIK3CA	7	0	0	n.m.
K13	0	no mutants	0	0	n.m.	n.m.



ccfDNA in localized and nonlocalized CRC patients

FFPE material of 21 of 22 Patients show feasible biomarkers for ccfDNA analysis. NGS sequencing procedure revealed results with high sensitivity. However, depending on the platform used it is a time consuming and cost intensive procedure. Digital PCR analysis (Bio-rad) could be shown to be an alternative strategy with a simple workflow when plasma samples were analyzed for known gene mutations. Results from tumor samples and corresponding liquid biopsies were shown for two variants of APC mutations (patient no. 1).

Summary/Conclusion

Circulating tumor DNA contain genetic alterations identical to those of the tumors themselves. Thus, genetic mutations detected in cancer tissues can be used as biomarkers for the analysis of plasma samples (liquid biopsy) from cancer patients.

The specificity and sensitivity of these liquid biopsies in stage IV disease patients is about 90-95%. For these patients liquid biopsy analysis may be suitable for tumor monitoring of neo-adjuvant or adjuvant therapy strategies.

Lower-stage patients with known genetic alteration in their tumor may also be monitored by analyzing liquids since the sensitivity and specificity is much higher than for classical serum based biomarkers as CEA or CA-125.

However, to date the use of liquid biopsy for tumor monitoring is based on the analysis of primary or metastatic tumor material to define feasible biomarkers for the individual patient.

Further improvement of the clinical work-flow of blood sampling and ccfDNA extraction is necessary to standardize these applications for routine diagnosis.
Furthermore, the appropriate choice of methods to detect mutated gene fragments with high sensitivity in plasma samples is still an open field and will be crucial for the introduction into routine diagnostic work flows.