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 (ctDNA) could become a promising biomarker in cancer care and prognosis. The use of ctDNA presents several conceptual advantages compared to classical gene expression analysis and tumor-biopsy sampling. ctDNA analysis is non-invasive and enables day-to-day patient follow-up and monitoring of treatment response. Analysis of ctDNA 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 ctDNA in a routine laboratory. Optimization and normalization of the Workflow of the pilot study covers all aspects of the respective procedure, starting with blood sampling, isolation of the ctDNA, determination of its concentration and determination of tumor-derived ctDNA and its fragmentation.

Work flow
Prior to analysis tumor derived DNA-fragments are enriched by cold-PCR and preparative gel isolation. The 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 specimens and mutations which are known to be frequently mutated in colon Cancer are sequenced by use of appropriate gene panels on an Ion Torrent Personal Genome Machine (PGM) or Ion Proton.

Methods
DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissues
DNA was isolated manually from one to 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 ctDNA extraction and sizing
Whole blood (10 ml) was drawn in a cell-free DNA blood collection tube (Sleek, 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 of each sample was determined (Qiubit 2.0, Life Technologies) and a diluted plasma DNA fragments was evaluated (Agilent Technologies, Santa Clara, USA).

Gene panels
A specific custom designed multiplex PCR panel was constructed using the AmpliSeq Design Tool (Life Technologies), the panel covers 520 genes: APC, TP53, KRAK, TPSA, SMAUG with 212 PCR fragments amplified simultaneously.

Ion Torrent PGM library preparation and DNA sequencing
Ion Torrent amplicon-typed 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, Life Technologies). Sample enrichment PCR, enrichment breaking, and enrichment were performed with the Ion PGM 100 Xpress Template Kit (Life Technologies). 100 ng of DNA was used to sequence barcoded samples on the Ion Torrent PGM or 60 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 software Torrent Suite to generate sequence reads, then trim adapter sequences, indices, indices, enzyme cuts and low-quality bases. After removal of low-quality reads, the high-quality reads were aligned to the human genome to identify mutations. The frequency of each sample, and P value (0.01) for all variants with a frequency of at least 1% were determined.

Quality and stability in serum and/or tumor
Blood samples either collected in normal EDTA tubes or in special blood collection tubes for stabilisation of cell-free plasma DNA (Cell-Free DNA Kit) were analyzed after 1, 3 and 6 days. DNA concentration in plasma was determined. DNA concentration in EDTA tubes increased according to storage time whereas DNA concentration in cell-free tubes remained constant indicating that blood cell loss was minimal and the amount of ctDNA was not diluted by genomic DNA from lipic 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 Bioanalyser System. Data show that the size of DNA-fragments isolated from sera is well below 200 bp by requiring special enrichment techniques.

Tumors are heterogeneous

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

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 80-90%. 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 alterations 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 workflow of blood sampling and ctDNA 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 inclusion into routine-diagnostic work-flows.