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Chapter 1

Introduction

1.1. Neuroblastoma

Neuroblastoma constitutes 7-10% of childhood cancers in Europe and the USA and approximately 15% of pediatric cancer deaths. It is the most frequent solid tumor in children and the most common solid tumor diagnosed during infancy [1]. Neuroblastoma is a disease of the sympathetic nervous system arising from the sympathoadrenal lineage of the neural crest. The migration of cells during the prenatal development explains the variety of locations the tumor can present. The majority of primary tumors are located in the retroperitoneal space, most frequently in the adrenal medulla. Neck, chest and pelvis represent further common sites of disease. Due to the dependency on the site of the primary tumor and the presence of potential metastases, the patient's symptoms can vary largely [2].

1.1.1. Clinical heterogeneity

Neuroblastoma is characterized by a large clinical heterogeneity: While a subset of tumors undergoes spontaneous regression or maturation, other tumors are much more aggressive and exhibit malignant behavior and rapid progression even despite multimodality therapy [1]. The International Neuroblastoma Staging System (INSS) represents a classification system of neuroblastomas into the risk categories low, intermediate and high according to the site of the primary tumor at diagnosis and the presence of metastatic disease. Furthermore, this system considers the resectability of the tumor [3]. However, even when the age of the patient is additionally taken into account [4], a reliable prediction of the tumor behavior from these considered parameters is frequently not possible.

1.1.2. Genetic alterations in neuroblastoma

Over the last decades, other molecular characteristics of the tumor have been shown to be predictive of patient outcome: Hyperdiploidy or near-triploidy of the tumor cells with gains affecting only whole chromosomes (numerical chromosomal aberrations, NCAs) is a predictor of favorable course of disease [5-7], especially in the absence of segmental chromosomal aberrations (SCAs) and amplification of the *MYCN* oncogene [8]. An association between these near-triploid tumors and low disease stages, lack of structural chromosomal aberration, young age of the patients and good outcome was stated [9]. In contrast to that, both a diploid DNA content and the presence of SCAs are frequently observed in advanced-stage tumors and associated with an unfavorable course of disease [8]. Frequently observed SCAs include deletions of chromosomes 1p and 11q [7, 10-13] or unbalanced translocations which lead to

gains of 17q [14, 15]. Further segmental alterations contributing to a poor prognosis of high-risk patients include deletions affecting chromosomes 3p and 4p as well as gains of 1q and 2p [16-19].

Amplification of the *MYCN* oncogene (MNA) is associated with poor prognosis and rapid progression in all disease stages. MNA was already report in 1984, when amplifications of the *MYCN*-locus of up to 140-fold were detected in neuroblastoma cell lines. When tumor tissue samples were analyzed, a large variation in the amplification levels could be determined with 3- to 10-fold higher levels of *MYCN* in 10 cases, and amplifications of even 100- to 300-fold in 12 cases. Furthermore, also a correlation between MNA and the tumor stage was observed: While 24/48 patients with advanced stages of disease showed amplification of *MYCN*, none of 15 analyzed patients with stage 1 and 2 disease were MNA-positive [20]. In a different study, only stage 1 and 2 patients were analyzed and also in this 'low risk' group, patients with MNA were associated with less favorable outcome compared to the MNA-negative low risk patients [21]. Amplification of *MYCN* is associated with poor outcome, even in patients below 18 months of age, even though the prognosis of this younger patient group is generally substantially better compared to older patients, also with the same stage of disease [22, 23].

In the risk classification of the International Neuroblastoma Risk Group (INRG), neuroblastomas are separated into localized tumors (stages L1 and L2) and metastatic tumors (stages M and MS) [24]. New staging strategies were needed to implement all available information on the clinical and biological characteristics of the tumor into the process of identifying the appropriate risk classification for the respective patient. Studies that consider the correlation of genomic aberrations with clinical parameters are, for example, the Low and Intermediate Risk Neuroblastoma European Study (LINES) of the SIOPEX group, where SCA data is already implemented into the therapy decision process.

1.1.3. Treatment

The methods used for the treatment of neuroblastomas include surgery, chemo-, radio- and antibody-therapy but also the sole and careful observation of the tumor and the patient's symptoms in certain cases. A crucial issue in the management of neuroblastoma is to assess if residual tumor cells remain in the patient's body after surgical resection of the tumor or after chemotherapy. The consideration of clinical as well as biological data is very important for the prediction of tumoral behavior. In some cases, adjuvant therapy is not needed despite the presence of residual tumor material, even at metastatic sites. In contrast to that, there are cases in which the biological characteristics reveal the need for adjuvant therapy even when the tumor is almost completely resected [2]. This highlights the importance of the present and

continuously improved staging systems to meet the immense clinical heterogeneity in neuroblastoma and assure the optimal patient care.

1.2. Circulating tumor DNA

1.2.1. Historical background

The presence of extracellular nucleic acids in the human blood was already described in 1948, 5 years before Watson and Crick described the helical structure of DNA [25]. The discovery remained unnoticed by the scientific community until around two decades later, when increased levels of DNA were detected in the sera of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis [26, 27]. In the late 1970s, cell-free DNA (cfDNA) was isolated and comprehensively characterized from the sera of healthy and diseased individuals. In this wide-ranging work, the authors not only described higher concentrations of DNA in the serum of patients with cancer compared to healthy individuals, but also in patients with metastatic diseases compared to patients with localized cancers. Furthermore, they stated a decrease in DNA of up to 90% following radiation therapy, and a correlation of therapy-mediated decreases in DNA concentrations and improved clinical outcome such as decreased tumor size [28]. After Stroun et al. confirmed the findings of higher extractable amounts of DNA in the cell-free plasma of patients suffering from different cancer types compared to normal controls [29], they were the first to show the cellular origin of cell-free DNA by detecting certain neoplastic characteristics, which were present in the corresponding tumor DNA, in the plasma of the respective patients [30].

Further tumor-specific genomic alterations, also observed in DNA isolated from the primary tumor, could be identified in DNA derived from the cell-free blood compartment of the respective patients in the 1990s. Several authors described the presence of mutated *RAS* genes such as *KRAS* [31-33] or *NRAS* [34], while other groups stated the feasibility to detect tumor-associated microsatellite alterations in the plasma/serum of patients with small cell lung cancer, head and neck cancer and clear cell renal carcinoma [35-37]. Furthermore, mutations in *p53* [38] and aberrant methylation of tumor suppressor genes [39, 40] were detected. The fraction of cfDNA, which is derived from tumor cells, is termed circulating tumor DNA or ctDNA.

A different field in the analysis of cfDNA was revealed by the discovery of fetal DNA in maternal plasma in 1997 [41]. Mean fetal DNA concentrations of 25.4 and 292.2 genome-equivalents/ml were stated in early (11-17 weeks gestation) and late (37-43 weeks gestation) pregnancies, respectively [42]. In the late 90s, genetic loci specific for the fetus such as Y-chromosome-specific markers in the case of a male fetus [43] and fetal *RhD* gene in the case of

rhesus D incompatibility [44] were shown to be useable for non-invasive diagnosis. Abnormal concentrations of fetal DNA in maternal plasma were linked to different pregnancy-disorders such as preterm labor [45] and preeclampsia [46], and have also been described in pregnancies complicated by fetal trisomy 21 [47]. Also epigenetic differences between maternal and fetal fraction of circulating DNA were stated in different studies [48, 49]. More recently, massive parallel sequencing was established for the detection of fetal trisomy 21, 18 and 13 in the maternal plasma [50-52]. Despite remaining technical issues and limitations due to the low fraction of fetal DNA in maternal plasma [43], this non-invasive diagnostic method provides the advantage of lack of risks for the fetus compared to other conventional prenatal diagnostic techniques such as chorionic villus sampling and amniocentesis [53].

1.2.2. Biology of ctDNA

The releasing process of tumor-derived nucleic acids into the circulation is still not fully elucidated. Two potential and not mutually exclusive mechanisms are suggested (Figure 1) [54]: In the passive release mechanism, DNA enters the circulation by cellular destruction of apoptotic and necrotic cells [55, 56]. While under normal circumstances, the thereby resulting debris are cleared by phagocytes leading to low levels of cell-free DNA [57], an accumulation of cellular debris and hence a higher release of DNA into the bloodstream occurs within a tumoral mass [58]. An active mechanism, suggesting spontaneous release of DNA into the bloodstream via microvesicles, has been reported from studies on different cultured cell lines [59-61].

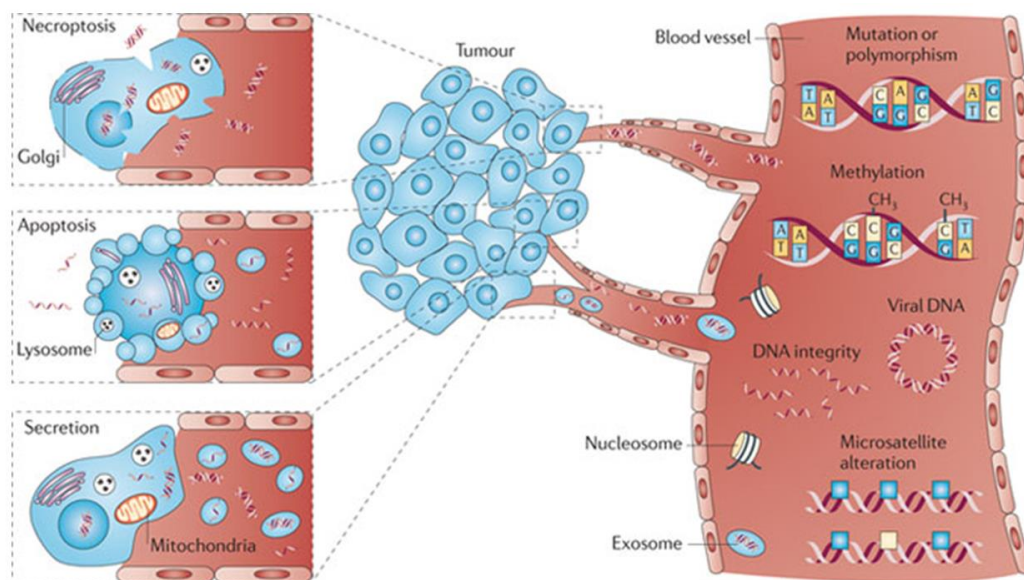


Figure 1: Possible release mechanisms of ctDNA into the human blood stream, taken from [54].

It was estimated that for patients with a tumor load of 100g (which corresponds to 3×10^{10} tumor cells), up to 3.3% of the tumor DNA is fed into the circulation every day [62]. The circulating DNA fragments are reported to consist of two different sized fractions with lengths of 100 to 200 base pairs and fragments of approximately 21 kilobases [56]. The length of the small sized fragments resembles the size of DNA wrapped around a nucleosome [63] and may therefore be the result of apoptotic DNA release due to degradation of cellular chromatin by a caspase-activated DNase [64, 65]. In contrast to that, the large fragments are most likely released from necrotic cells [56]. Notably, ctDNA, i.e. cell-free DNA derived from tumor-cells, accounts for only a fraction of total cfDNA in the circulation. It was stated that the ctDNA-fraction shows a high degree of variation and can range between 0.01% and 93% [56, 66].

Mouse experiments revealed a very short half-life of circulating nucleic acids when purified DNA was injected into the animals' blood stream [67, 68]. A study on hemodialysis patients also led to a very short determined half-life of 4 min [69], whereas the half-life of circulating fetal DNA in the maternal plasma was stated to be approximately 16 min [70]. While experiments concerning the clearance mechanism of cell-free DNA in mice showed that DNA/anti-DNA complexes were cleared from the circulation via the liver [71], a different study suggested clearance of cell-free DNA by the kidney, because subcutaneously injected bacteriophage lambda DNA could be isolated in the urine of mice and Y-specific DNA sequences were detectable in the urine of women after receiving a blood transfusion from a male donor or being pregnant with a male fetus [72].

1.2.3. Analysis of ctDNA

Quantitative analysis of cfDNA

The first quantification of cell-free DNA was performed by Leon et al. who stated higher cfDNA concentrations in cancer patients compared to healthy controls [28], an observation which was also made by other groups when analyzing patients with various cancer types [73-76]. However, although cancer patients reveal higher levels of cfDNA than healthy controls, great variations in the amount of DNA in plasma and serum of both groups were stated [54].

Leon et al. also described significantly higher amounts of DNA in the serum of patients suffering from metastatic disease compared to patients with localized cancers [28]. More detailed studies focusing on this theme were performed later on, when higher plasma DNA concentrations were detected in lung cancer patients with stage 4 disease compared to patients with localized disease [73], or when elevated cfDNA levels were observed in prostate cancer patients with lymph node and distant metastases while the concentrations in patients with localized prostate cancer were found to be similar to male and female healthy controls [75].

Different groups compared the DNA amount in the cell-free blood compartment of non-small cell lung cancer (NSCLC) patients with different stages of disease: Gautschi et al. stated a significant correlation between advanced tumor stage and increased amounts of plasma DNA [77] and in a very recent study by Newman et al., a novel and extremely sensitive targeted sequencing approach called CAPP-sequencing enabled the detection of tumor-derived circulating DNA in 100% of patients with at least stage 2 NSCLC, but only in 50% of patients with stage 1 disease [78]. In a large study, a total of 223 patients suffering from breast, colon, pancreas or gastroesophageal cancer were analyzed and differences in the frequency of detectable levels of ctDNA between patients with localized (stage 1-3) and metastatic disease (stage 4) were observed in each of the four cancer types (Figure 2A). When 640 patients with various cancer types were analyzed in the same study, the detectability of ctDNA correlated strongly with distinct stages: tumor-specific sequences were only detectable in the plasma DNA of 47% of patients with stage 1 cancers of any type, whereas the detection frequency rose to 55%, 69% and 82% for patients with stage 2, 3 and 4 cancers, respectively (Figure 2B) [79].

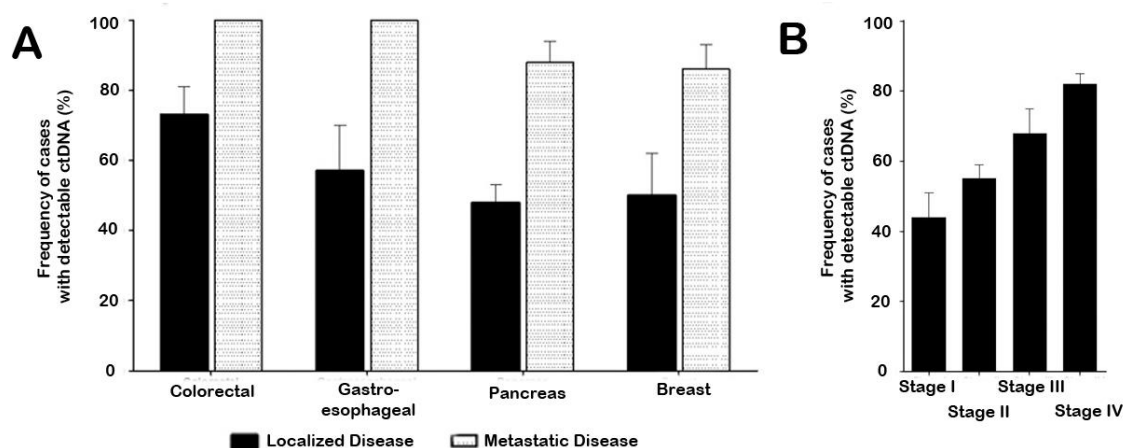


Figure 2: ctDNA in patients with localized and metastatic disease (A) detectability of ctDNA between patients with localized stages (stage 1-3) and metastatic disease (stage 4) (B) relationship between detectability of ctDNA and different stages of cancer (various cancer types), modified from [79].

Interestingly, the fractions of patients with detectable tumor-specific circulating DNA varied among different tumor types. While in patients with advanced cancers of bladder, colon, esophagus and ovaries, ctDNA was detectable in all analyzed subjects, less than 10% of patients with gliomas harbored detectable levels of ctDNA (Figure 3). This suggests that the blood-brain barrier may act as physical filter that hinders DNA fragments from entering the bloodstream [79].

Increased amounts of plasma DNA were detected in patients with SLE and rheumatoid arthritis [26, 27], but are also present in individuals with inflammation [80], trauma [81], stroke [82], after exhaustive exercise [83] or in elderly patients suffering from acute or chronic illnesses [84]. These findings add a further layer to the variability in the concentration levels of cell-free DNA which makes it altogether unfeasible to draw clear conclusions from the sole determination of the cfDNA-concentration at a single time

point. Furthermore, it seems not reasonable to compare cfDNA levels between different patients, even within one tumor entity. Instead of that, quantitative analyses of cell-free DNA may only be useful when comparing different time points within the course of disease of a single patient thus not analyzing the level of cfDNA at a given time point but rather the kinetics.

The levels of circulating DNA are not constant but under fluctuation, which was shown by the analysis of cell-free DNA in pregnant women, where, interestingly, the maternal fraction fluctuated to a higher extent than the fetal fraction [85]. In cancer patients, cfDNA concentrations were shown to vary within the course of disease: Changes in the amounts of circulating DNA in plasma and serum were shown to correlate with tumor burden and therapy response. This phenomenon was already stated in 1977, when decreases in the plasma DNA concentration of up to 90% were observed upon radiotherapy. A correlation

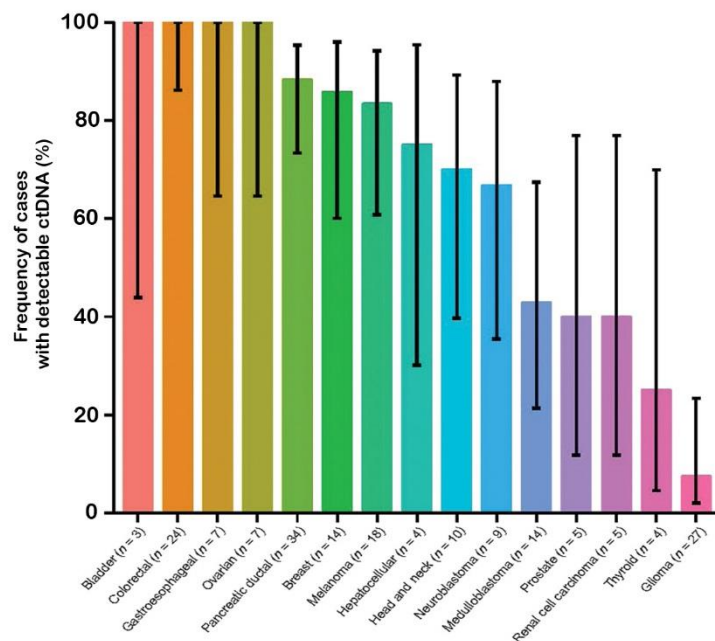


Figure 3: Fraction of patients with advanced malignancies and detectable ctDNA, taken from [79].

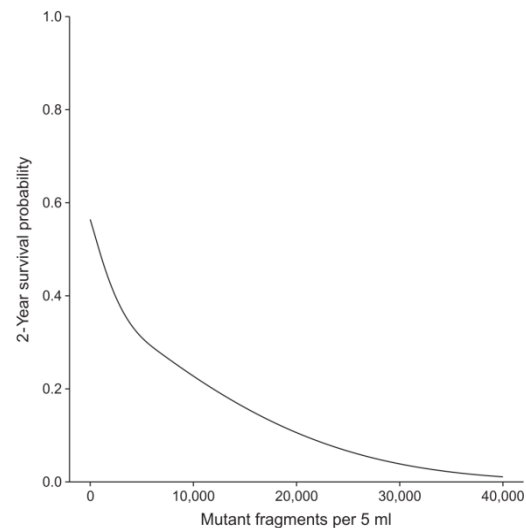


Figure 4: Correlation between ctDNA concentration (measured in mutant fragments per 5 ml) and 2-year survival, taken from [78].

between decreasing DNA concentrations and improved clinical conditions such as decreasing tumor size or reduction of pain was observed, whereas stable or even increasing DNA concentrations indicated a lack of response to the treatment [28]. These findings were affirmed in studies on patients with lung cancer [74, 86], ovarian carcinomas [87], and NSCLC [77]. Bettgowda et al. suggested a prognostic value of ctDNA by showing that metastatic colorectal cancer (CRC) patients with low levels of circulating tumor-derived DNA lived significantly longer than patients with higher levels [79] (Figure 4). This correlation between cfDNA concentration and survival was also observed in ovarian cancer patients, where elevated plasma DNA levels were found to be an independent predictor for disease-related death (Figure 5) [88].

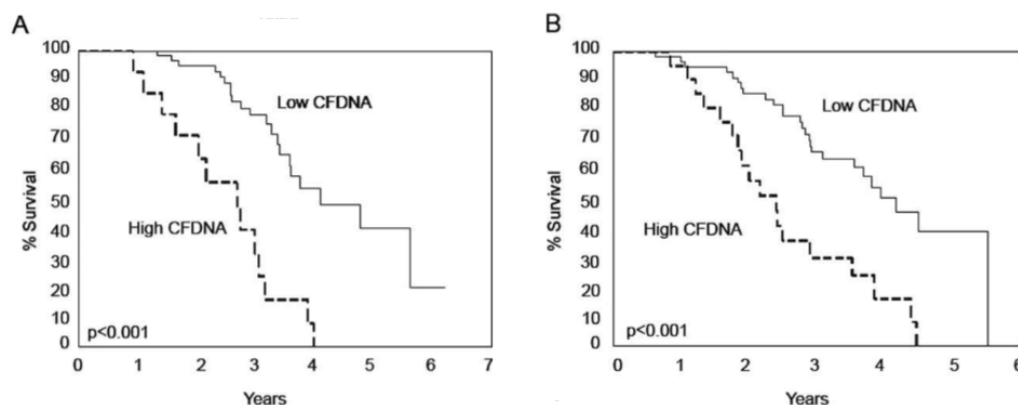


Figure 5: Kaplan-Meier curve for survival of ovarian cancer patients with high levels of cfDNA, i.e. $\geq 20,000$ genome equivalents per ml (dotted line), compared to patients with lower levels (full line) (A) training set (B) validation set; taken from [88].

Diehl et al., in turn, highlighted the importance of post-operative ctDNA levels in CRC-patients by revealing a significant difference in recurrence-free survival between patients with detectable versus undetectable ctDNA levels at the first follow-up after surgery (Figure 6) [66]. In a very recent study, the levels of ctDNA were compared to radiographically measured tumor volumes and therapy response in NSCLC patients. Thereby, a correlation between ctDNA levels in pretreatment samples and the tumor volume measured by CT and PET imaging was observed (Figure 7A). Furthermore, plasma samples from different patients with advanced NSCLC receiving different therapies were analyzed and again, ctDNA levels strongly correlated with tumor volumes at the different time points during therapy (Figures 7B and 7C) [78].

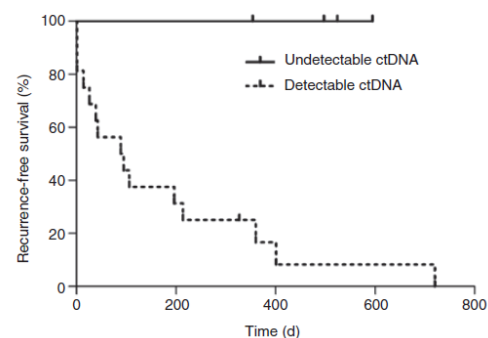


Figure 6: Correlation between post-operative ctDNA detectability and recurrence-free survival, taken from [66].

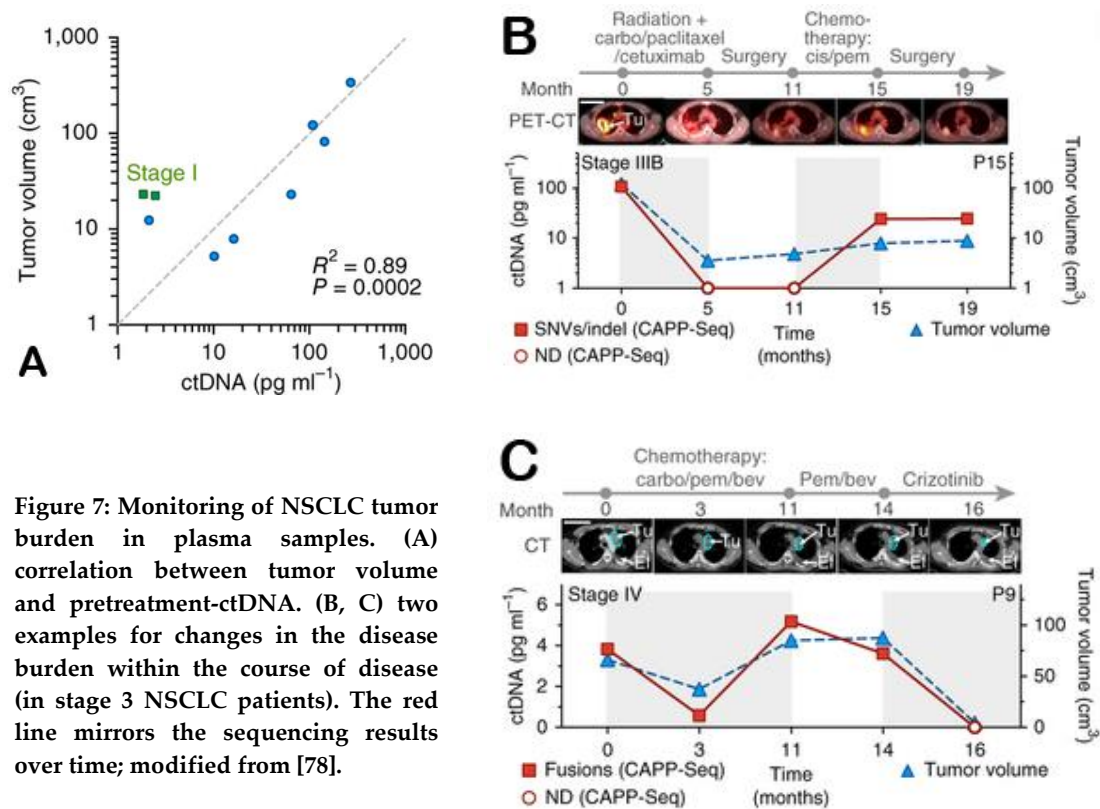


Figure 7: Monitoring of NSCLC tumor burden in plasma samples. (A) correlation between tumor volume and pretreatment-ctDNA. (B, C) two examples for changes in the disease burden within the course of disease (in stage 3 NSCLC patients). The red line mirrors the sequencing results over time; modified from [78].

Qualitative analysis of tumor-specific alterations

There are two main approaches in the qualitative analysis of ctDNA: The identification of specific tumor-associated alterations is carried out via PCR-based approaches or targeted sequencing, while whole-genome analyses allow the identification of gains and losses of whole chromosomes or parts thereof. Both strategies have advantages and disadvantages which will be discussed in detail below.

It seems to be no coincidence that the oncogene *KRAS* was the first chosen target for the detection of tumor-specific alterations in cell-free DNA. It is among the genes with the highest mutation frequency found in cancer with mutations located in three mutation hotspots. The defined mutation hotspots facilitated the development of tests with high specificity and sensitivity. The tumor suppressor gene *p53* represents a further gene suitable for the analysis of specific gene mutations, as it is frequently mutated in a large number of tumor types and associated with tumor progression [89]. In addition to these two genes, PCR-based assays were also developed to identify clinically relevant mutations in, for example, *BRAF* and the epidermal growth factor receptor (*EGFR*) [90, 91] in the plasma and serum. cfDNA offers the advantage that once the patient's mutation status is assessed, these mutations can be monitored

during treatment and the course of disease without the need of repeated biopsies. However, a key problem in these approaches is that the mutations have to occur with high frequency and at specific genomic positions. A further downside is that wild-type sequences often represent a problem in mutation assays due to low levels of tumor-specific sequences in a large background of unmutated sequences [54]. Different groups established PCR-assays to specifically enrich mutant sequences in the *KRAS* gene and to detect one mutant sequence in up to 10,000 wild-type copies. The first method to achieve this sensitivity level was based on PCR primers creating a restriction site specifically in the wild-type DNA that is subsequently cut by a restriction enzyme. This step was followed by a second round of amplification where only the uncut mutant sequence was enriched [92, 93]. The second methodological approach was based on mutant-specific primers that only amplify DNA sequences containing the defined mutation [94]. The reliable detection of *BRAF* mutations such as V600E, which is present in over two thirds of metastatic melanomas, has become more and more important with the development of new anti-*BRAF* drugs [95, 96]. Although such site-specific approaches are very promising for the detection of mutated genes and the monitoring thereof, sensitivity and specificity are two key problems that need further validation to determine the true clinical utility of these assays.

Genome-wide analyses of cfDNA, in contrast, are enabled by array techniques such as array comparative genomic hybridization (aCGH) [63] or next generation sequencing (NGS) approaches which facilitate the detection of gains/amplifications or losses of genetic material. Whole-genome sequencing additionally allows the visualization of mutations at the nucleotide level or translocation events. High-resolution array techniques are frequently used due to their cost effectiveness while still providing a high level of information. Genome-wide approaches are required for the identification of NCAs and SCAs, where gains and losses affect whole chromosomes (NCAs) or parts thereof (SCAs). Due to the frequent occurrence of chromosomal copy number changes in human cancer, approaches allowing the mapping of changes in the copy number status in a genome-wide manner are very important. Heitzer et al. performed aCGH analyses on plasma DNA of patients with advanced CRC and were able to determine genome-wide estimations of copy number changes using an array platform with over 55,000 oligonucleotides [63]. Several groups used massive parallel sequencing techniques for the identification of copy number alterations in patients suffering from hepatocellular cancer, CRC or breast cancer [97-99]. The general workflow of pan-genomic ctDNA analyses is illustrated in Figure 8 [100]. In both depicted approaches, i.e. aCGH and NGS, total cfDNA (consisting of DNA fragments derived from both tumor cells and normal cells) is isolated from the plasma/serum of cancer patients. The copy number status is assessed by counting the number of aligned DNA fragments in relation to their position in the genome. The number of normal cell-derived DNA fragments is theoretically constant across the entire genome while the tumor-specific fragments may show a variation and thereby reflect the copy number alterations of

their tumorous origin. With the help of bioinformatics tools, the number of reads at a given location in the genome is converted into a copy number status.

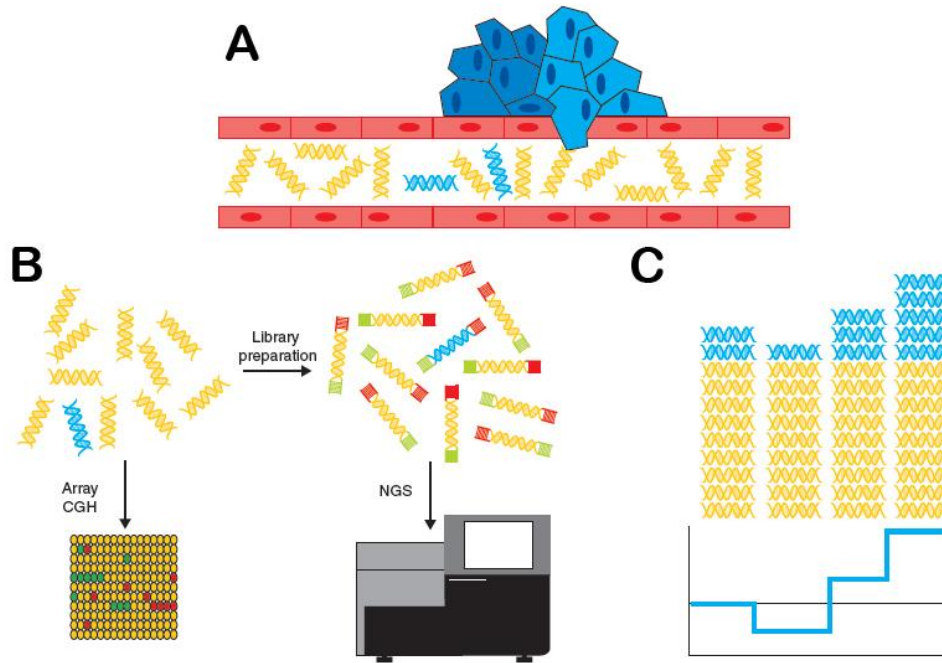


Figure 8: General workflow of different genome-wide approaches for ctDNA analyses. (A) ctDNA fragments (light blue) and DNA fragments released from normal cells (yellow) are present in the circulation of cancer patients. (B) Isolated blood plasma DNA is either directly subjected to an array (aCGH) or a library is prepared for subsequent NGS analysis. (C) After alignment, DNA fragments are counted and the number of reads is converted to a copy number status (blue line); modified from [100].

A combined approach of site-specific and pan-genomic analysis was chosen by Heitzer and coworkers in 2013; they performed whole-genome sequencing from cfDNA of prostate cancer patients with a shallow sequencing depth to assess the genome-wide copy number status, and additionally used a targeted sequencing approach with high coverage on a panel of 55 genes of interest as well as 38 introns including frequent fusion breakpoints [101].

Taken together, site-specific approaches offer the advantage that small fractions of mutated sequences can be detected, even when they are masked by a large number of wild-type sequences due to the dilution of tumor-specific DNA fragments by cfDNA derived from non-tumorous cells. Limitations to these analyses of tumor-specific mutational regions are that the mutations of interest have to be known in advance and that they have to occur in defined mutational hotspots to be targetable via targeted sequencing or PCR-based approaches. If the mutation sites are not known, a biopsy has to be taken and sequenced to assess the mutations and a patient-specific assay has to be designed and used to analyze follow-up samples [102].

Due to the high cost, whole-genome or whole-exome approaches on the other hand usually lack the high levels of sensitivity provided by site-specific analyses but are in turn extremely useful for the identification of NCAs and SCAs and the determination of the copy number status across all chromosomes. A further advantage is that no previous knowledge on tumor-specific alterations is needed.

1.2.4. Clinical applications of ctDNA

Genotyping – tissue versus liquid biopsy

Although tumor tissue represents the gold standard for genetic analyses, some well-known challenges concerning acquisition and utility of tissue remain, which may limit the informational content of this approach. Biopsies are not only uncomfortable, invasive procedures that provide risk for the patient, but are in addition often not available in case of inoperable tumors [57]. Tumor heterogeneity probably represents the major challenge of tissue biopsies, a problem which was highlighted by studies on the genomic landscape of various cancer types. In these studies, a large genetic diversity within the tumor was revealed [103, 104]. Vogelstein et al. proposed four different types of tumor heterogeneity which are depicted in Figure 9: Intratumoral heterogeneity refers to genetic differences among the cells of one tumor due to new mutations being acquired during cell divisions. Cells with a large spatial distance within the tumoral mass will therefore show more genetic differences than neighboring cells [105]. The second type of heterogeneity is intermetastatic heterogeneity, which describes differences between two metastatic lesions within a particular patient. It is common for metastatic lesions to have up to 20 genetic alterations not shared by other metastatic sites [105]. This can be explained by the fact that the founder cells of the metastasis, i.e. the cells that separated from the primary tumor and formed the metastatic lesion, may arise from distinct locations of the primary tumor and therefore harbor different mutations that set the starting point of the mutational landscape of the respective metastasis [106]. Intrametastatic heterogeneity, in contrast, refers to heterogeneity among the cells of an individual metastasis. Each metastatic lesion harbors a set of founder mutations and new mutations are acquired with each cell division as it grows. Although the founder mutations may be targetable by antitumor agents, the newly acquired mutations may be the cause for resistance mechanisms. In addition to that, metastatic lesions are generally rarely removable by operation. The fourth type of heterogeneity in cancer is interpatient heterogeneity, which describes differences in the tumor genomes between different patients [106].

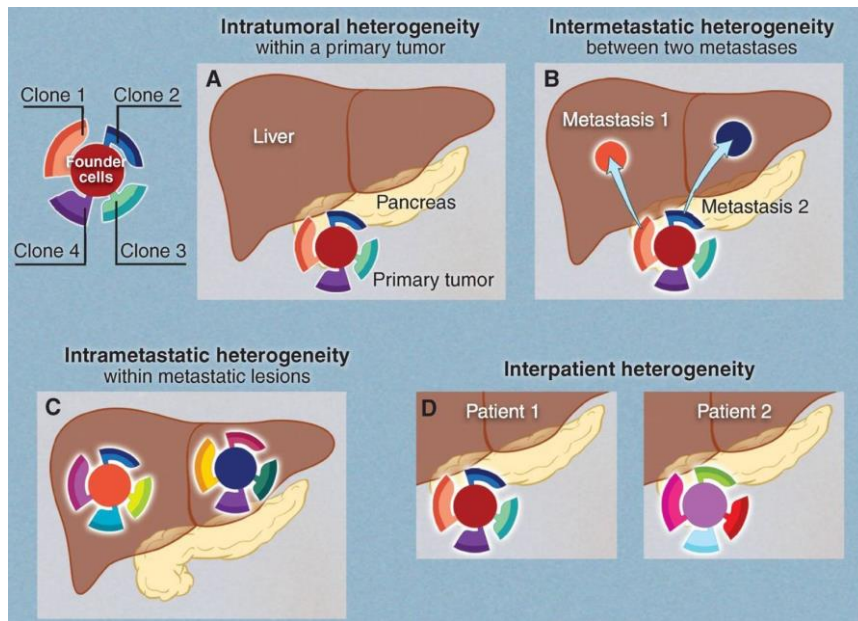


Figure 9: Different types of tumor heterogeneity. In this model, the primary tumor is located in the pancreas while the metastatic lesion is located in the liver, taken from [106].

The aspect of intratumor heterogeneity makes it understandable that sampling of a single region of the tumor may limit the extent of obtained information on the tumor mutations. In addition to that, repeated sampling of tumor tissue is not feasible which limits the possibility to investigate the tumor evolution during the course of disease [107].

Liquid biopsies, i.e. Analyses of circulating tumor DNA and circulating tumor cells (CTCs), may overcome the mentioned challenges. The sampling itself is non- or minimally-invasive and, in contrast to surgeries, virtually risk-free. Repeated sampling during the course of disease is feasible which opens up new doors in the investigation of tumor dynamics. Thereby, the genetic information obtained from ctDNA analysis should in theory not only cover the informational value of tumor biopsies, but the challenge of tumor heterogeneity may also be overcome as ctDNA may represent an average picture of the tumor genome. Different groups agree that the somatic mutations assessed by ctDNA analyses widely represent the underlying tumor genome and can therefore provide an alternative to tumor sampling without loss of information. Higgins et al. showed the feasibility of analysing plasma-derived cell-free DNA in order to detect *PIK3CA* mutations in patients suffering from metastatic breast cancer. Due to the fact that the mutational status of *PIK3CA* can change during the course of disease, they highlighted the importance of repeated assessment of this gene, which is feasible by repeated blood sampling and analysis of ctDNA [108]. Similar studies were also performed in patients suffering from other cancer types such as ovarian cancer and small cell lung cancer and also thereby, cancer mutations which were present in the tumor, were detectable in the DNA extracted from the plasma of the respective patients [35, 109]. Thierry et al. compared the mutational status of

KRAS and *BRAF* obtained from tumor tissue with the plasma DNA of metastatic CRC patients. The analysis of cfDNA showed 100% for both specificity and sensitivity for *BRAF*V600E and over 90% specificity and sensitivity for seven tested *KRAS* mutations. These results led to the suggestion that cfDNA analysis could replace tumor-section analysis [110].

In contrast to the studies in which single mutations detected in the tumor were compared with the mutational status derived from the cell-free DNA, Chan et al. performed genome-wide analyses of copy number aberrations and identified point mutations in the cell-free blood compartment of cancer patients via massive parallel sequencing. They compared pre- and postoperative ctDNA samples from four hepatocellular carcinoma patients to the corresponding primary tumors as shown in Figure 10. In the Circos plots, which depict the copy number status across the whole genome, it can be seen that characteristic copy number alterations present in the primary tumor (innermost ring) were also detected in the plasma samples obtained before treatment, which are shown in the middle ring. For all cases, these copy number changes almost disappeared after resection of the tumor (post-operative plasma in the outer ring) [97].

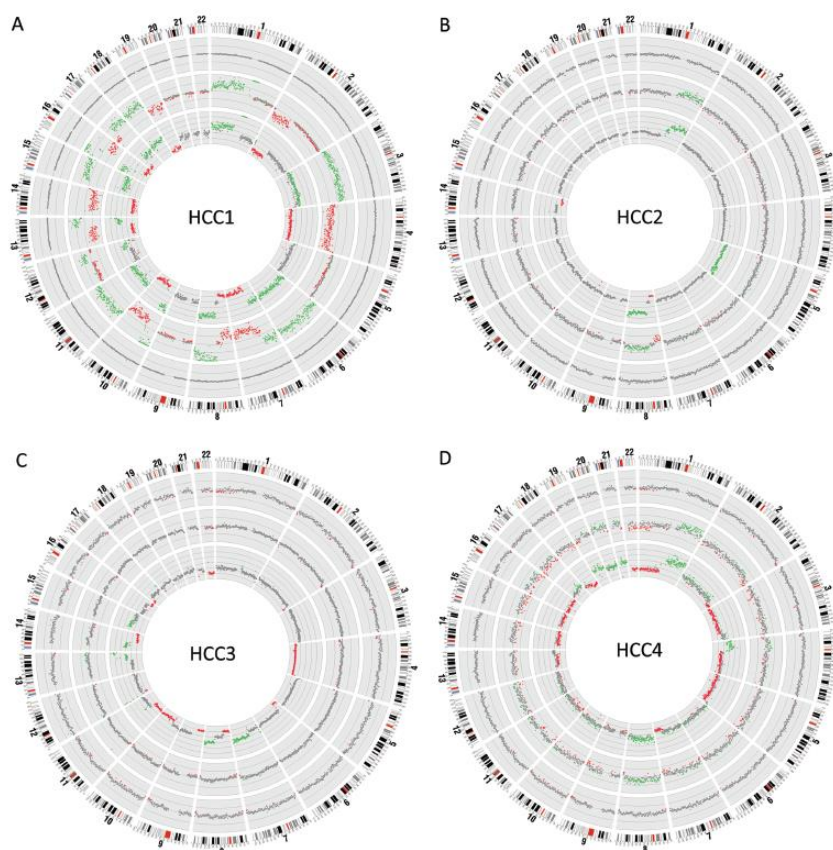


Figure 10: Copy number status from four hepatocellular carcinoma patients. Inner ring: tumor tissue, middle ring: presurgery plasma sample, outer ring: postsurgery plasma sample; taken from [97].

In the same study, the plasma DNA of a patient suffering from synchronous breast and ovarian cancers was analyzed and aberrations specific for both cancer types were found to mix up in the circulation [97]. This phenomenon was also stated by Heitzer et al. who compared copy number changes detected in the plasma DNA of CRC patients to the copy number status in the primary tumor and the metastases of the particular patients. Thereby, aberrations specific for both the primary tumor and the metastatic lesion were present in the circulation [63]. These findings are in concordance with other groups proposing that ctDNA analysis may be the key to overcome molecular heterogeneity because of DNA fragments from all tumorous sources in the patient's body mix together in the circulation [111].

Monitoring tumor burden and therapeutic responses

The status of a patient's tumor burden is usually assessed by imaging techniques or the analysis of circulating biomarkers. Prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9 and CA-125 are examples for such protein biomarkers. Unfortunately, many malignancies lack reliable biomarkers and even the markers used in the daily routine are often characterized by low levels of specificity - an elevation is not necessarily linked to tumor growth or progression [57]. The presence of ctDNA is very specific for a patient's tumor. The analysis of ctDNA is furthermore advantageous due to the shorter half-life of DNA fragments in the blood [66] compared to that of protein biomarkers [112], a difference that may lead to a more "real-time" assessment of the tumor burden. Dawson et al. evaluated the potential of ctDNA compared to other circulating markers (CA 15-3 and CTCs) to monitor the tumor burden and thereby the response to treatment. ctDNA was revealed to be a more sensitive biomarker of disease burden and show a greater dynamic range than the other analyzed biomarkers. Increases in ctDNA levels were detectable up to several months before radiological methods indicated an increase in the tumor size. In addition to that, ctDNA levels were proposed as useful indicator of prognosis due to an observed association with patient outcome [99]. In a different study on CRC patients, specific rearranged fragments were identified in each individual patient via massive parallel sequencing. Those rearrangements were subsequently tracked via PCR reactions with specific primers spanning the breakpoints. In this manner, a ratio of arranged vs. non-arranged fragments could be determined and measured during the course of disease. The results for one patient can be seen in Figure 11, where the amount of tumor DNA in the plasma decreased after surgery, increased again during the following days and dropped after chemotherapy and resection of a secondary tumor in the liver [113]. As aforementioned, Newman et al. assessed ctDNA concentrations using a targeted sequencing approach and correlated their findings to the tumor volume obtained from imaging techniques

in NSCLC patients. Thereby, they were able to detect a very strong correlation between those two parameters during the course of disease [78].

The timing of blood collection in relation to the treatment may be a crucial factor that requires consideration when monitoring tumor burden and treatment response. In mouse experiments, an

increase in ctDNA release was observed immediately after treatment which may be an early indicator of occurring death of tumor cells and response to treatment. In contrast, plasma samples collected shortly before the administration of therapy may reflect the real overall tumor burden [114].

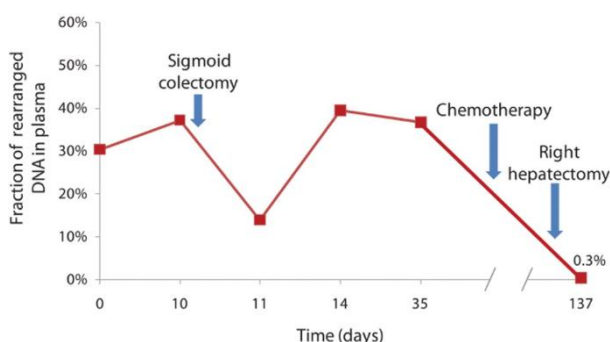


Figure 11: Levels of cell-free DNA fluctuating in a colon cancer patient's blood plasma during treatment, taken from [113].

Early detection of treatment resistance and residual disease

The development of resistance to anticancer therapies represents a key problem in cancer management. Resistances arise due to an evolving spectrum of somatic mutations in genes and pathways within the tumor that render the therapeutic agents ineffective. The increased acquisition of additional mutations occurs due to the fact that chemotherapy and targeted agents put the tumor under high selective pressure. The mutations leading to resistance are often found in a very small fraction of the initial tumor which gets larger until the cells of the small fraction are the dominant clone as the tumor evolves and thus evades treatment [107]. Acquired resistances upon treatment with anti-*EGFR* therapies are well studied in patients with CRC and lung cancer. Anti-*EGFR* drugs such as gefitinib or erlotinib become ineffective in approximately 50% of lung cancer patients due to an acquired amino acid substitution (T790M) in the kinase domain, which increases the binding of *EGFR* for ATP and therefore hinders the binding of the inhibitor [115]. Recent studies have demonstrated the high potential of ctDNA analysis for the early detection of such arising resistance mechanisms. Oxnard et al. demonstrated the feasibility of detecting *EGFR* mutations in plasma samples of lung cancer patients treated with erlotinib, and stated that *EGFR*T790M mutations were detectable in the plasma up to 16 weeks before progression was radiographically manifest [116]. Several different groups studied the possibility to detect acquired resistance mechanisms in the blood of CRC patients upon anti-*EGFR* therapy. Misale et al. observed *KRAS* mutant alleles being present in

the plasma up to 10 months before disease progression was radiographically detectable. As can be seen in the exemplary data shown in Figure 12, an acquired *KRAS* mutation was detectable in the plasma of a CRC patient several months before the size of the liver lesion increased and also prior to the elevation of CEA levels in the blood [117].

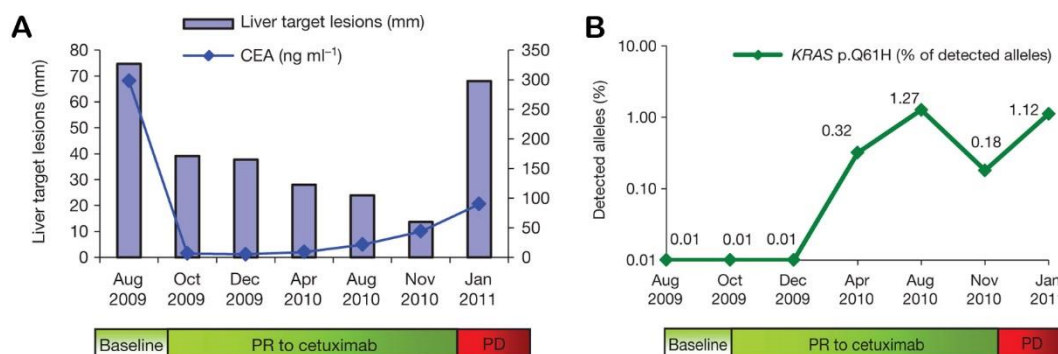


Figure 12: Detection of acquired *KRAS* mutations in the plasma DNA of a patient treated with cetuximab (A) blue bars show size of liver lesion and blue line mirrors CEA-levels over time. (B) levels of detected mutated *KRAS* alleles during the course of disease. Abbreviations: PR, partial response; PD, progressive disease; modified from [117].

DNA extracted from plasma samples of CRC patients treated with anti-*EGFR* therapy was also analyzed by a different group using a combination of whole genome sequencing at a shallow sequencing depth and deep sequencing of a set of genes associated with acquired resistances. The development of resistance was thereby found to be associated with gains of *KRAS* as well as amplification of other genes such as *MET* and *ERBB2* [118].

A further potential application of ctDNA could be the early detection of remaining tumor cells in the patient's body after surgery or therapy, i.e. minimal residual disease (MRD). These residual tumor cells can lead to recurrence of the tumor, and up to now there is no possibility to reliably detect whether all cancer cells are gone after surgery/treatment. Due to this lack in effective detection methods, a large number of patients is treated with high-risk adjuvant chemotherapy without knowing if they are probably already cured [57]. Circulating tumor DNA may be a useful marker of MRD when measured after surgery before the administration of adjuvant therapy. Diehl et al. analyzed the plasma of CRC patients after undergoing resection of the tumor. They determined the mutational status of each patient by analyzing the resected tumor and searched for the thereby assessed mutations in the post-operative blood samples. The patients were observed for 2 to 5 years and ctDNA was shown to be a sensitive marker of MRD: All patients where postoperative ctDNA was detectable in the blood, experienced recurrence while the patients with no remaining detectable levels of ctDNA stayed disease-free [66].

1.3. ctDNA analysis in Neuroblastoma

Several groups analyzed if circulating tumor DNA may be of diagnostic or prognostic value in neuroblastoma patients. Combaret et al. conducted the first study regarding this matter: They assessed the status of *MYCN* via PCR-amplification from serum and plasma samples of neuroblastoma patients with and without *MYCN* amplified tumors. Thereby, elevated *MYCN* copy numbers were detected in 31/32 and 1/70 patients with and without MNA, respectively. In addition to that, no amplification of *MYCN* was detected in 72 control samples from healthy donors. Real-time quantitative PCR led to the results that *MYCN* sequences in the blood of neuroblastoma patients with MNA was 25-600 fold higher than in other patients or healthy controls [119]. Gotoh et al. used real time quantitative PCR to quantify *MYCN* together with a reference gene in order to assess possible *MYCN* amplifications in the serum of patients with known *MYCN* status. The ratio between *MYCN* and the reference was significantly higher in patients with MNA compared to the non-MNA group. Both sensitivity and specificity of this approach were 100% when a cut-off between amplified vs. non-amplified *MYCN* status was set at 10 fold higher *MYCN* levels compared to the reference gene [120]. It was therefore shown in those two studies that the *MYCN* status can be reliably assessed in the cell-free blood compartment in the majority of neuroblastoma patients.

The determination of the *MYCN* status in the plasma/serum was taken a step further in 2009, when Combaret et al. analyzed the influence of the stage of disease on the detectability of MNA in the serum. For patients with stages 4, 4S or 3, sensitivities of 85%, 83% and 75%, respectively, were found while the specificity of the analysis was 100% in those stages. However, the sensitivities for patients with stage 1 and 2 disease were low. It was therefore shown that the detection of MNA in the serum depends on the cancer stage and that the assay may be of high potential in stage 3 neuroblastoma and patients with metastatic disease [121].

In 2011, two groups demonstrated the feasibility of assessing the allelic status of 17q and 11q, two frequently affected chromosomes in neuroblastoma, by analysis of circulating DNA. 17q gains were determined by quantifying two distinct gene regions on 17q in proportion to a reference gene located on 17p by using a quantitative PCR-approach. This analysis was performed on serum and plasma samples of neuroblastoma patients with known 17q status. In patients younger than 18 months, the assay showed a specificity of almost 95% even though the sensitivity was only about 60%. In older patients, however, specificity and sensitivity were lower with approximately 70% and 50%, respectively [122]. A similar study on the assessment of 11q loss from serum samples was performed, in which loss of genomic material was identified by microsatellite analysis. A significant difference could be observed between patients with and without 11q loss [123]. The two studies suggest, that the allelic statuses of the

q-arms of chromosomes 17 and 11 can be assessed by ctDNA analysis, but further studies are needed to confirm the reliability and utility of such assays.

In a very recent work by Combaret et al., the detectability of the mutational status of *ALK* in serum and plasma samples was observed. A very sensitive PCR-approach was used to identify alterations in three known mutational hotspots within the *ALK* gene. The results of this study revealed sensitivities of at least 85% and specificities of at least 91% for the three analyzed hotspots. The assay therefore represents a reliable method to assess *ALK* mutations in the three observed regions [124].

Sausen et al. investigated the potential to use ctDNA to predict MRD and relapse: Personalized rearrangement biomarkers were used to track ctDNA in plasma samples of four neuroblastoma patients during a MRD-immunotherapy trial. Tumor-specific DNA was thereby detected in two patients who relapsed. In the two other patients, no ctDNA was detected and the two patients were without relapse at least 4 years later at the last follow-up. The study demonstrates that there is a promising potential for ctDNA as sensitive and specific marker of MRD and relapse [125].

1.4. Aims of the study

Due to the fact that no genome-wide analysis of ctDNA was performed so far in pediatric patients in general and in neuroblastoma in particular, this study aimed to investigate the feasibility of such an approach. The present work was restricted to stage M (former stage 4) neuroblastoma patients.

Both peripheral blood (PB) plasma and bone marrow (BM) plasma samples were analyzed to verify if both sources can be used. This comparison was facilitated by the availability of bone marrow plasma left-overs from the diagnostic routine in the laboratory.

The first aim of the study was to determine the concentrations of cell-free DNA per ml plasma and to investigate whether the level of circulating tumor DNA in these samples is sufficient to perform pan-genomic analysis.

The feasibility of isolating sufficient tumor-specific DNA from 1 ml plasma to identify tumor-specific genomic aberrations by Ultra High Density SNP-array analysis should be investigated.

In the second part of the study, the aim was to correlate the detected numerical and segmental chromosomal aberrations with those detected in the primary tumor or in the bone marrow-derived disseminated tumor cells (BM-DTCs) in those cases in which data from different tumor cell sources were available.

Furthermore, the aberrations detected in the peripheral blood plasma should be compared to those detected in the bone marrow plasma (in cases where both specimens were available at the

same time point) in order to analyze whether all bodily fluids harbor the identical cross-section of the tumor genome.

Chapter 2

Manuscript

“Genome-wide analysis of liquid biopsies
reveals a novel layer of tumor heterogeneity in
neuroblastoma”

Genome-wide analysis of liquid biopsies reveals a novel layer of tumor heterogeneity in neuroblastoma

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Sophia C. Huetter: Conception and design, development of methodology, acquisition of data (plasma collection, DNA extraction, SNP array analyses), analysis and interpretation of data

Diana S. Walder: Technical support (plasma collection)

Clemens Brunner: Technical support (SNP array analyses)

M. Reza Abbasi: Acquisition of data (BM-DTCs)

Fikret Rifatbegovic: Administrative and technical support

Inge M. Ambros: Conception and design, analysis and interpretation of data, review of the manuscript

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Abstract

Purpose: Tumor genome analysis is crucial for neuroblastoma patients to determine prognosis and optimal treatment strategies. Diagnostic genome analysis is frequently limited by insufficient tumor tissue and intra-tumor heterogeneity. Isolating cell-free tumor DNA from more easily accessible patient biomaterial sources could overcome these challenges. We investigated the feasibility in neuroblastoma patients of cell-free peripheral blood (PB) and bone marrow (BM) plasma for tumor genome analyses.

Experimental Design: Cell-free DNA was isolated from 123 plasma samples from 22 stage M neuroblastoma patients, and ultrahigh-density SNP array data from 16 samples were compared with data from the corresponding tumor or disseminated tumor cells from bone marrow (BM-DTCs).

Results: Genomic aberrations were retrieved from 11/16 cfDNA samples. The majority of genomic aberrations detected in cell-free tumor DNA from our patient cohort were concordant aberrations detected in other tumor cell sources. While genomic aberrations detected in cell-free tumor DNA from BM plasma or BM-DTCs were identical (3/3), discordances were detected between PB plasma and tumor tissue (1/1) sources.

Conclusions: Cell-free tumor DNA may serve as an additional source for tumor genome analyses, not only in cases for which bioptic or surgical interventions carry a high risk for the patient, but especially to detect additional genetically distinct tumor clones. Our findings contradict the widely accepted view that all bodily fluids yield an identical picture of tumor genetics. Comparing datasets from different intra-patient locations will produce a more complete representation of tumor heterogeneity, thus, improving our understanding of tumor dynamics and progression in individual patients.

Statement of translational relevance

Intra-tumoral heterogeneity represents a major limitation to tumor genome analysis, which is crucial for optimal patient care in NB as this relies, to a very large extent, on the analysis of genomic markers. The analysis of cftDNA in bodily fluids, i.e. liquid biopsy, may provide a solution to overcome this problem. In this study, we report the feasibility of performing genome-wide analysis from as little as 1 ml of PB- and BM-plasma from stage M neuroblastoma patients. By comparing cftDNA from those bodily fluids to other tumor cell sources, we have discovered the high potential of liquid biopsies being quite representative of the underlying tumor tissue. However, we have also detected differences between cell-free DNAs derived from PB- and cell-free DNAs derived BM-plasma samples indicating a further layer of heterogeneity in NB patients which contradicts the widely accepted view that all bodily fluids harbor the identical genetic cross-section of the tumor burden. These data can pave the way for new diagnostic strategies based on cell free nucleic acids.

Introduction

Neuroblastoma, the most common extracranial solid tumor in childhood, accounts for 7-10% of childhood cancers in the USA and Europe and for about 15% of pediatric cancer deaths. It is a tumor arising from the sympathoadrenal lineage of the neural crest with most primary tumors occurring retroperitoneally, most frequently in the adrenal medulla. Further common sites of disease include the thorax as well as neck and pelvis [1, 2]. Neuroblastoma tumors show a large heterogeneity in clinical and biological characteristics and behaviors: They may regress or mature spontaneously, reflecting induction of apoptosis or differentiation [3], or exhibit extremely malignant behavior with rapid progression, sometimes even despite intensive therapy [4]. It has been shown that hyperdiploid or near-triploid tumors with numerical chromosomal aberrations (NCAs), i.e. whole-chromosome gains, are associated with a more benign disease behavior and favorable patient outcome [5, 6] while segmental chromosomal aberrations (SCAs) such as deletions at the short arm of chromosome 1, at the long arm of chromosome 11 and/or gains of 17q correlate with an unfavorable course of disease [5-8].

Amplification of the *MYCN* oncogene, a frequent event in neuroblastoma, is in most instances associated with advanced stage of disease and poor prognosis even in infants [9, 10], although the outcome of neuroblastoma patients less than 18 months of age is, in general, substantially better than the outcome of older patients with the same stage of disease [11, 12]. Current staging strategies have been defined to take account of the correlation of genomic aberrations with clinical parameters [13] and different studies, such as, for example, the Low and Intermediate Risk Neuroblastoma European Study (LINES) of the SIOPEN group, have started to implement SCA data into the process of defining the optimal treatment for the patient.

So far, stage M neuroblastomas have not been categorized into different clinical/genetic groups in patients over 18 months. However, currently available data suggest that also in stage M tumors different entities can be defined according to their genomic profile. Tumors with amplification of the *MYCN* gene represent a substantial genetic sub-group of metastatic neuroblastomas with a frequency of about 30% (IM Ambros, pers. communication). In addition to this amplification, also amplification of *ALK* and different genes on the long arm of chromosome 12 (e.g. *MDM2*) are described [14-16]. The general hallmark of not *MYCN*-

amplified tumors is the presence of SCAs. Whether certain aberrations, such as deletions at the long arm of chromosome 11, have an impact on clinical subgroups, needs verification in larger patient cohorts [5]. However, additional genomic events may help to sub-classify these tumors. The list of mutated/deleted genes in neuroblastoma is rather limited and encompasses mainly genes involved in the chromatin-remodeling process with mutations and/or intragenic deletions of *ATRX* and *ARID1A/ARID1B* being present in about 6% and 11% of analyzed tumors, respectively, while *ALK* has been found to be affected in 9.2% followed by mutations in *MYCN*, *PTPN11* and *NRAS* in less than 3% of analyzed cases [17-20].

The exact determination of the tumor genetics at diagnosis is therefore indispensable. Unfortunately, single tumor biopsies do not necessarily lead to the desired information due to extensive intratumoral heterogeneity (ITH), as shown by a large number of authors (e.g. [21]). Different studies have described this phenomenon also in neuroblastoma, identifying markers such as 1p status, *MYCN* copy number and ploidy [22-25]. Recently, two manuscripts describe temporal ITH as an increase of the mutational burden over time for genes involved in the *RAS-MAPK* pathway and of *CHD5*, 9p loss and *DOCK8* [26, 27]. Although the analysis of tumor tissue is still the gold standard for investigating tumor genetics [28], current data indicate that also disseminated tumor cells (DTCs) in the bone marrow can serve as excellent source for tumor genome analyses in neuroblastoma patients [29]. However, less invasive techniques capable of capturing tumor heterogeneity are needed to explore the best strategy to identify the most relevant clones that may be invisible when analyzing only a single biopsy.

The presence of circulating tumor DNA (ctDNA) in the circulation of cancer patients is well described, especially in patients with metastatic disease [30]. Due to the fact that we use DNA derived from PB-plasma as well as from BM-plasma, we introduce the term cell-free tumor DNA (cftDNA) for the tumor-specific DNA fraction.

The total DNA in the plasma, i.e. DNA derived from tumor-cells as well as from non-tumor cells, is referred to as cell-free DNA (cfDNA).

The tumorous origin of ctDNA was proven by the detection of tumor-specific genomic alterations in the plasma of cancer patients that matched the corresponding primary tumors (e.g. tumor-specific microsatellite alterations, aberrant promoter hypermethylation of tumor suppressor genes or mutations in *RAS*-genes) [31-33].

The mechanisms responsible for the release of cell-free DNA has still not fully been elucidated, but active secretion of DNA into the circulation via microvesicles as well as passive mechanisms such as necrosis and apoptosis and combinations thereof have been suggested [34, 35].

Several studies were undertaken to investigate ctDNA in neuroblastoma. Elevated *MYCN* copy numbers were detected in the cell-free blood compartment of patients with known *MYCN*-amplified tumors [36, 37]; the mutational status of *ALK* [38] as well as the allelic status of 11q [39] and 17q [40] were also determined in the circulation of neuroblastoma patients. Furthermore, Sausen et al. stated a promising potential of cfDNA as surrogate for the level of clinical disease and predictor of minimal residual disease (MRD) and relapse [18].

However, the key challenge remains technical because tumor-derived cfDNA may only make up a very small fraction of cell-free DNA in the body [35]. The sensitivity of the detection method used therefore determines the level of detection of ctDNA in liquid biopsies [41].

Our study was designed to investigate the concentrations of totally cell-free DNA in the peripheral blood (PB)- and bone marrow (BM)-plasma samples from stage M neuroblastoma patients and to determine whether the levels of cftDNA in these samples are sufficient for pan-genomic analysis. We tested the feasibility of performing UHD SNP array analysis on 1 ml plasma to identify tumor-specific genomic aberrations and compared the different SNP array results from individual patients obtained from different tissues/sources such as BM-derived (DTCs) vs. cftDNA from PB-plasma as well as from BM-plasma or tumor tissue vs. plasma cftDNAs.

Materials and Methods

-Samples

123 samples were obtained from 22 stage M neuroblastoma patients. Both peripheral blood- (n=52) and bone marrow- (n=71) derived plasma samples were analyzed to test for suitability of tumor genome analyses. DNA was extracted from tumor tissue and BM-derived DTCs.

- Plasma collection

Plasma was collected from whole blood samples – left-overs from routine diagnostic procedures – as described by Heitzer et al. [42] or by centrifugation at 1600 g without prior fixation. Plasma from BM-samples sent in for routine tumor cell quantification was collected from the upper fraction after density gradient centrifugation using Lymphoprep™ (Axis-Shield PoC, Oslo, Norway) and further centrifugation to avoid cellular contamination. 1 ml aliquots from the cell-free plasma-supernatant were stored at -80°C or used immediately for cfDNA isolation.

- Isolation and quantification of cfDNA

cfDNA was isolated from 1 ml plasma using the ChargeSwitch® gDNA 1 ml Serum Kit (Invitrogen, Waltham, Massachusetts, USA), a purification method based on magnetic beads that provides a switchable charge on a solid surface for the capture of nucleic acids by variation of the pH level. The protocol provided by the manufacturer was specifically adapted to the respective requirements: The Lysis Mix including 700 µl of ChargeSwitch® Lysis Buffer and 30 µl of Proteinase K for 1 ml plasma was added and the samples were incubated at room temperature for 60 minutes. After the addition of 250 µl ChargeSwitch® Purification Buffer and 30 µl ChargeSwitch® Magnetic Beads, the samples were incubated for 2 minutes at room temperature followed by 60 minutes on a magnetic rack. The pellets were washed according to the manufacturer's protocol and DNA was eluted in 30 µl ChargeSwitch® Elution Buffer. The Invitrogen ChargeSwitch® gDNA 1 ml Serum Kit was compared to the QIAamp® DNA Blood Mini Kit (Qiagen, Venlo, Netherlands), a column-based technique which was used for 1 ml plasma samples according to the manufacturer's instructions with an elution volume of 30 µl. The concentrations of isolated cfDNA were measured using the Qubit® 2.0 fluorometer

together with the Qubit™ dsDNA High Sensitivity (HS) Kit (both Life Technologies, Carlsbad, California, USA).

- Tumor samples and BM-DTCs

DNA was extracted from tumor tissue and BM samples by applying the “high salt” extraction technique. In case of BM samples, DNA was directly extracted from cytospin slides or enriched by a magnetic bead-based technique [29]. The tumor cell infiltration rate was determined with the AIPF technique and reported as number GD2⁺/CD56⁺ cells per million mononuclear cells (MNCs) [43].

- SNP-array analyses and visualization of SNP array data

The Cytoscan HD (Affymetrix Inc., UK Ltd) platform was used for tumor genome analyses of tumor tissues, BM-DTCs and cfDNA from PB- and BM-plasma. For further information on the interpretation of SNP-array data see Ambros et al. and the analysis of DTCs see Abbasi et al. [16, 29]. The method is based on hybridization of the labelled sample DNA to an array containing 1.9 million non-polymorphic markers and over 740 thousand SNP markers with an average intergenic and intragenic marker spacing of 1737 bp and 880 bp, respectively. The procedure was carried out exactly according to the manufacturer’s recommendations, the arrays were scanned with the GeneChip scanner (Affymetrix Inc., UK Ltd) and the resulting data were analyzed using the ChAS software (Affymetrix Inc, UK Ltd), a visualization tool with the possibility to analyze single chromosomes and parts thereof separately.

- Circos plots

The combined visualization of different SNP array results in one graph was achieved by a Circos plot visualization tool [16].

RESULTS

- cfDNA concentrations and sample characteristics

123 plasma samples from 22 neuroblastoma patients were examined. The median cfDNA concentration in the analyzed samples was 193.2 ng/ml (IQR = 541.7 ng/ml). The cfDNA concentrations were significantly higher in the BM-plasma samples as compared to PB-plasma samples (median concentrations 420.0 ng/mL vs. 15.4 ng/mL, respectively, $P < 0.0001$). Overall, the cfDNA concentration in the patient-derived plasma samples was significantly higher as compared to 4 PB-plasma samples from healthy donors (median concentrations 193.2 ng/mL vs. 6.4 ng/mL, respectively, $P = 0.004$).

SNP array analysis was performed on cfDNA from 16 plasma samples on which 11 samples revealed tumor-specific aberrations and 5 samples showed a “flat profile” without detectable gains or losses, which indicates the exclusive presence of normal-cell DNA, or that the majority of the cfDNA was derived from normal cells masking the tumor cell-derived DNA. To better understand the factors influencing the proportion of cftDNA within the total cfDNA (table 1), we compared the detectability of genomic aberrations in the cfDNA to the disseminated tumor cell infiltration rate and, in a second approach, to the cfDNA concentrations in the plasma. Interestingly, the presence of genomic aberrations, providing evidence of cftDNA, did not correlate with the cfDNA concentration in the plasma or the input amount of DNA for SNP array analysis. However, the presence of cftDNA (from PB or BM), as deduced by the presence of genomic aberrations, correlated significantly with the amount of DTCs in the bone marrow (Fig. 1).

Table M1: Characteristics of samples on which SNP array analysis was performed

Sample	Sample origin	DNA in plasma (ng/ml)	DNA-Input for SNP analysis (ng)	BM-infiltration rate (GD2 ⁺ cells per 10 ⁶ MNCs)	Aberrations detected by SNP (yes/no) ^a
1	BM	2,194.3	256.0	700,000	yes
2	BM	15.8	2.43	185	no
3	BM	650.0	104.0	0	no
4	BM	315.0	63.0	250,000	yes
5	BM	1,510.0	302.0	800,000	yes
6	BM	882.0	147.0	75,000	yes
7	BM	573.0	95.5	750,000	yes
8	PB	109.3	8.2	400,000	yes
9	PB	278.8	30.2	n.a.	no
10	PB	110.4	13.8	250,000	yes
11	PB	6.1	1.22	n.a.	yes
12	PB	75.6	6.3	n.a.	yes
13	PB	48.5	9.7	375,000	yes
14	PB	460.0	92.0	64	no
15	PB	562.1	89.0	75,000	yes
16	PB	90.6	15.1	n.a.	no

^a aberrations detected by SNP: yes = tumor-specific aberrations detected, no = „flat“ (disomic) profiles
 abbreviations: BM, bone marrow; PB, peripheral blood; n.a., no data on BM-infiltration rate available

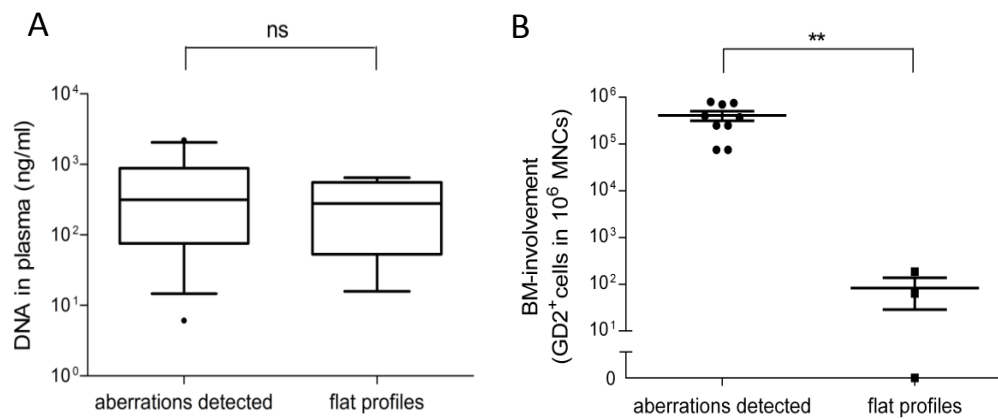


Figure M1: Dependency of cfDNA-detectability on: A) cfDNA-concentrations in the plasma (aberrations detected n=11, flat profiles n=5) ($P=0.617$, Mann-Whitney test) and B) the BM-involvement at the time point of sampling (GD2⁺/CD56⁺ cells per million mononuclear cells (MNCs)). In 9 cfDNAs, neuroblastoma-typical aberrations were detected, and in three samples, only a flat profile was found. ($P=0.009$, Mann-Whitney test).

- Isolation method of cfDNA

Two different extraction kits, the Qiagen QIAamp® DNA Blood Mini Kit and the Invitrogen ChargeSwitch® gDNA 1 ml Serum Kit, were compared to identify the optimal method for cfDNA isolation from plasma. Both kits were applied on 10 paired BM-plasma samples. The cfDNA yield was significantly higher (average fold change of 2.50) using the ChargeSwitch Kit as compared to the conventional extraction technique (Fig. 2 and Supplementary Table S1).

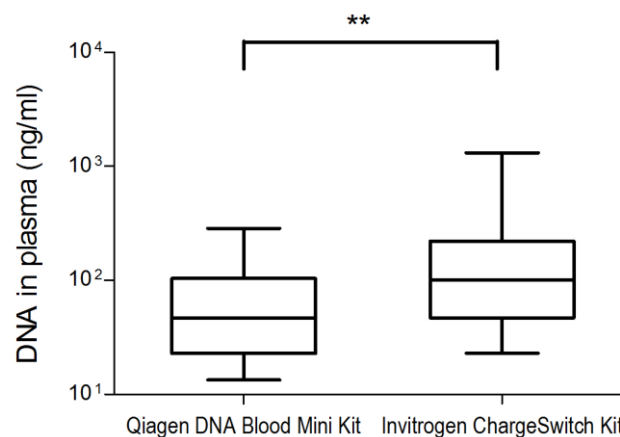


Figure M2: Circulating cell-free DNA concentrations (y-axis) of patient samples (n=10) extracted with the ChargeSwitch extraction technique as compared to the conventional extraction procedure ($P = 0.006$, Wilcoxon matched-pairs signed rank test).

- Tumor genome analyses

Tumor genome analyses using SNP arrays enable high resolution insights into the human genome by providing information not only on copy number changes but also on the allele status. It is therefore a very powerful method for detecting deletions/gains/amplifications affecting large chromosomal segments or even single genes or parts thereof. In addition, information on loss of heterozygosity (LOH) with or without copy number changes - the latter known as copy-neutral LOH (cnLOH) - can be obtained.

Although the analysis of UHD SNP array data on a single chromosome level (as enabled in the ChAS software) is indispensable, Circos plots are a very useful tool to compare two or more samples in a genome-wide manner. In all Circos plots presented in this paper, the chromosome ideograms with the Mb information are located along the outside, followed inwards by the copy number probe tracks of BM-derived DTCs and cftDNA from BM-plasma (Fig. 3). In Fig. 4,

these rings are followed by cftDNA from PB-plasma and the tumor tissue after chemotherapy in the innermost ring. The copy number track is color-coded with black dots indicating disomic regions while blue color mirrors gain or amplification of chromosomal material and red dots indicate loss of material.

SNP array results from BM-derived DTCs and cftDNA isolated from BM-plasma from the same time point were compared: In Fig. 3A and Supplementary Table S2, data from a 4-year-old neuroblastoma patient (female, stage M, 70% BM-infiltration rate) is shown. The time point of sampling was 4 days after the beginning of cytotoxic chemotherapy (Carboplatin, VP-16 and Vincristin). The detected NCAs and SCAs including all breakpoints are completely identical in the two samples. In Fig. 3B and Supplementary Table S3, data from a 2-year-old neuroblastoma patient (male, stage M, 80% BM-infiltration rate) at the time point of diagnosis is shown. In this case, again, all detected aberrations are completely identical in the two samples.

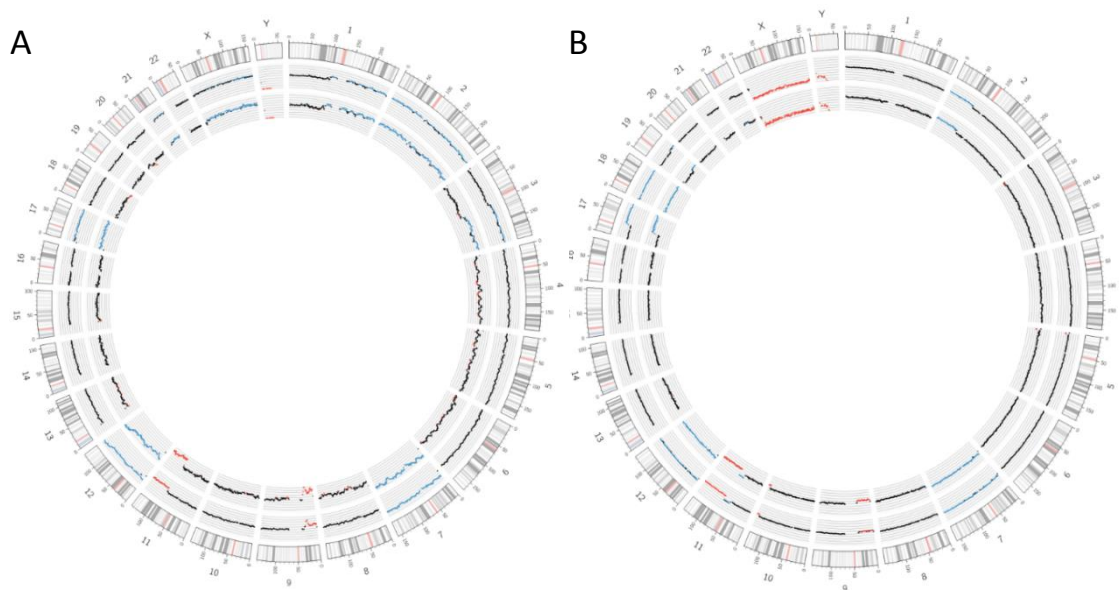
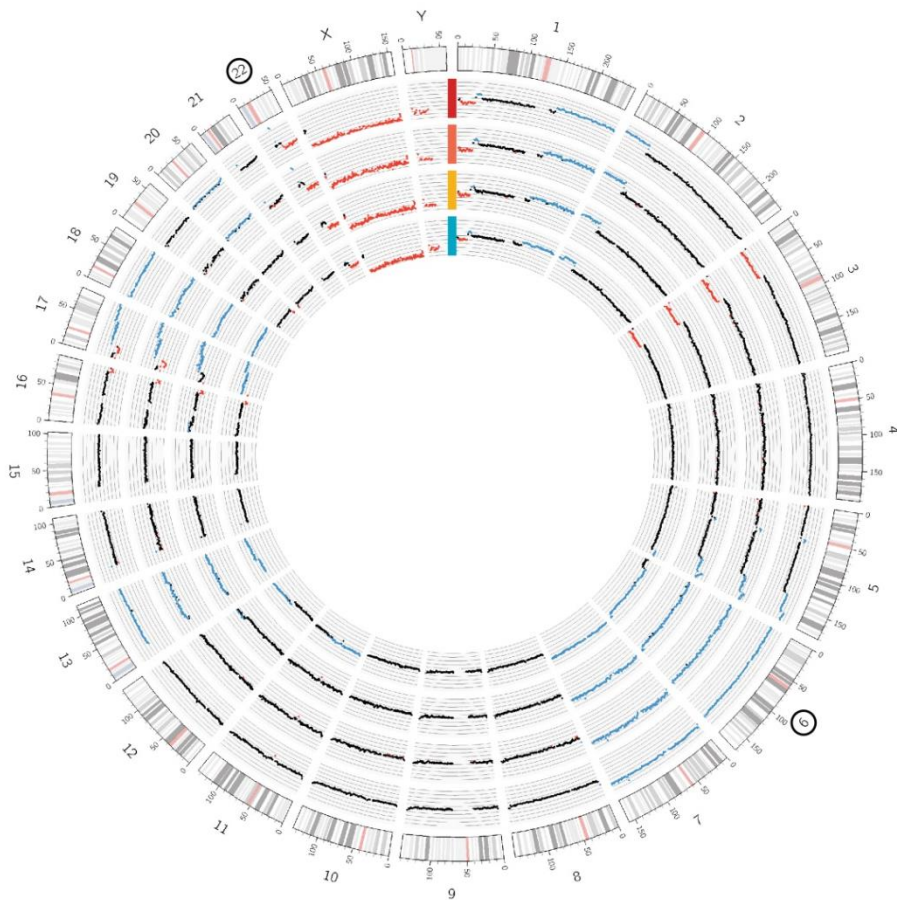


Figure M3: Circos plots of BM-derived DTCs and BM-plasma-derived cftDNAs from two different patients. From outside inwards: chromosome ideograms and distances in Mb, copy number tracks of BM-derived DTCs, copy number tracks of cftDNA from BM-plasma. All NCAs and SCAs and all breakpoints are shared between the two samples of each patient. (A) NCAs of chromosomes 2, 7, 17, 21, 22, X; SCAs on chromosomes 1, 3, 5, 9, 11, 12 (see also Supplementary Table S2). The seeming discordances in single areas between the two profiles (on chromosomes 4, 6, 8, 13, 15, 18, 20) are due to waviness of the cftDNA and do not represent real copy number changes as verified by the detailed ChAS software analysis. (B) NCAs of chromosomes 7, 12, 18; SCAs on chromosomes 2, 3, 4, 5, 9, 10, 11, 17, 19, 22 (see also Supplementary Table S3).

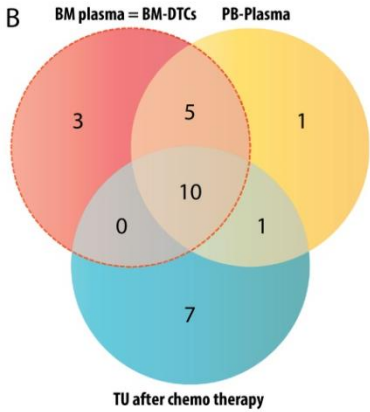
Tumor genome analysis was also performed on four different samples from a further patient (male, 4 years old, stage M, BM-infiltration rate 10% and 40% on right and left side, respectively) (Fig. 4). The 3 samples depicted in the outer rings of the Circos plot (i.e. BM-DTCs, cftDNA from BM-plasma, cftDNA from PB-plasma) were collected at the time point of diagnosis; the profile of the tumor tissue, which was resected after chemotherapy 5 months later, is shown in the innermost ring (Fig. 4A and Supplementary Table S4). In concordance with the two cases shown in Fig. 3, completely identical NCAs and SCAs, including the identical location of all breakpoints, were detected in BM-derived DTCs and cftDNA from the BM-plasma. However, the different samples are not completely concordant concerning their aberrations. While 10 breakpoints are shared between all samples (as depicted in the Venn diagram), 7 breakpoints are present in the tumor tissue post therapy only. 1 breakpoint is only present in the cftDNA from PB-plasma and 3 breakpoints were only detected in the two BM-derived samples. 1 breakpoint is shared between the tumor tissue and PB-plasma and 5 breakpoints are shared between the 3 samples at the time point of diagnosis, i.e. BM-derived DTCs and cftDNAs from BM- and PB-plasma. Notably, no breakpoints are shared between BM-derived samples and tumor tissue that are not present in the cftDNA from the PB-plasma (Fig. 4B).

Figures 4C and 4D show the SNP array profiles of two exemplary and highly interesting chromosomes in more detail: Most notably, an amplicon on chromosome 22 is present in BM-derived DTCs and BM-plasma cftDNA but not in the other 2 samples, which means that it was not even detectable in the PB-plasma which was sampled at the same time point. A second breakpoint on the same chromosome, however, is shared between all 4 samples (Fig. 4C). A breakpoint on the p-arm of chromosome 6 is present in the tumor tissue only, while a gain in the q-terminal region is shared between the tumor tissue and cftDNA from PB-plasma, but is not present in the two BM-derived samples (Fig. 4D).

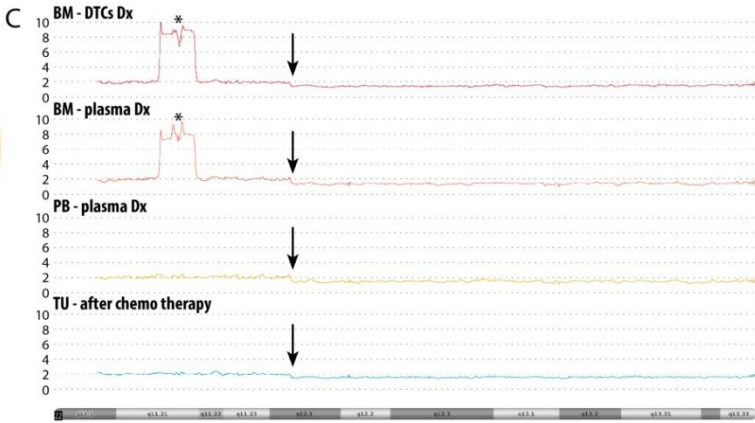
A



B



C



D



Figure M4: A) Circos plot of four different samples from one patient. From outside inwards: chromosome ideograms and distances in Mb, copy number tracks of BM-derived DTCs, cftDNA from BM-plasma, cftDNA from PB-plasma (all sampled at diagnosis) and tumor tissue (resected 5 months later). Black dots mirror disomic regions, higher copy numbers are shown in blue, while red dots mirror loss of genomic material. Chromosomes 6 and 22 are highlighted: An amplicon on chromosome 22 is only present in the two BM-derived samples while a further breakpoint is shared between all 4 samples (for details see Fig. 4C). On chromosome 6, the 3 samples from the time point of diagnosis share a whole chromosome gain that does, in contrast, not span across the whole chromosome in the tumor tissue sample, whereas a segmental gain in the q-terminal region is only shared between the tumor tissue and PB-plasma (for details see Fig. 4D). B) Venn diagram showing shared breakpoints as well as unique breakpoints in the respective samples. Note that BM-derived DTCs and cftDNA from BM-plasma are depicted as one set in the diagram due to their 100% concordance. C) and D) show the SNP array profiles for two exemplary chromosomes. The smooth signal which mirrors the copy number state is shown above the ideogram. C) Detailed view of the q-arm of chromosome 22: An amplicon from 20.2 to 22.0 Mb (maximum copy number ~10, marked with asterisks) is shared between BM-derived DTCs and cftDNA from BM-plasma but is not present in the other samples while a breakpoint at 27.0 Mb (see arrows) separating the disomic region from a segmental loss of genomic material is shared between all 4 samples. D) Detailed view of chromosome 6: while the 3 diagnostic samples (i.e. BM-DTCs and cftDNAs from BM- and PB-plasma) share a whole chromosome gain with a copy number of ~2.5, this gain does not stretch across the whole chromosome 6 in the tumor tissue (see arrow, breakpoint at 22.6 Mb). A segmental gain (marked with asterisks, spans from 164.3 Mb to q-terminus) is shared between the tumor tissue and cftDNA from PB-plasma, but was not detected in the two BM-derived samples. See Supplementary Table S4 for detailed information on tumor-specific aberrations and locations of breakpoints.

Discussion

Cell-free DNA has attracted growing interest in oncology due to its variety of clinical applications: Data from different studies indicate that genetic changes detectable in ctDNA are widely concordant with the underlying tumor genome (e.g. [44]) and that ctDNA may therefore serve as a non-invasive alternative to tumor sampling. Chan et al. analyzed ctDNA from hepatocellular carcinoma patients and were able to achieve a genome-wide profiling of copy number aberrations and point mutations. When comparing their findings to the corresponding tumor, they detected a high concordance between those samples and therefore a high potential of ctDNA for tumor genome analyses [44]. Similar studies were undertaken to analyze ctDNA also in different cancer entities. Heitzer et al., for instance, established genome-wide tumor-specific copy number alterations from cfDNA of colorectal cancer patients [42]. The analysis of circulating DNA might further be useful for monitoring the tumor burden of a patient [45] as well as for the early detection of treatment resistance and recurrence (e.g. [46]).

With this report, we present the feasibility of performing whole genome analysis in neuroblastoma patients by analyzing circulating DNA obtained from as little as 1 ml of cell-free plasma. A crucial factor in working with cell-free DNA is the identification of the optimal isolation technique for the extraction of DNA from plasma samples. The desired technique needs to offer high reproducibility, which can be achieved by the correct choice of commercially available kits, and should yield high amounts of good quality DNA. We compared a broadly used kit, the QiaAmp DNA Blood Mini Kit, to a technique based on binding of DNA to magnetic beads at a specific pH and subsequent elution of the purified DNA upon pH variation. Surprisingly, the latter method yielded significantly higher amounts of DNA in our paired BM-plasma sample experiments. The validation of the different extraction techniques was crucial for our study, as it is usually not possible to collect large volumes of blood from neuroblastoma patients for routine diagnostic procedures due to their generally young age. We need to emphasize that virtually all plasma samples used in this study were left-overs from routine PB or BM tests. Overall, cell-free DNA was isolated from 123 plasma samples obtained from 22 stage M neuroblastoma patients. Our aim was to establish whether the concentrations of cell-

free DNA in 1 ml of plasma samples are sufficient for pan-genomic analysis via UHD SNP arrays and whether tumor-specific aberrations could be identified in this way. The analysis was performed on 16 of the before-mentioned plasma samples from neuroblastoma patients leading to the detectability of tumor-specific aberrations in 11 samples. The 5 remaining samples, showing “flat profiles” without detectable gains or losses are most likely the result of a large proportion of cell-free DNA being derived from normal cells and thereby masking the tumor-specific fraction. A huge variability in the fraction of ctDNA was already stated previously with a proposed range of 0.01 to 93% tumor-specific DNA in the total cfDNA [35, 47]. The detectability of tumor-specific DNA did not correlate with the total amount of DNA in the respective plasma sample, which highlights the differences in the cfDNA amounts between single patients and the fact that no clear conclusions can be drawn from the sole determination of the DNA concentration in the plasma. This is in accordance with the fact that although several groups stated higher amounts of DNA in patients with cancer compared to healthy individuals, large variations in both groups were observed (e.g. [48]). In contrast, a significant relationship could be observed between the presence of tumor-specific DNA, as shown by the presence of tumor-typical aberrations in the cell-free blood or BM compartment, and the infiltration rate of DTCs in the BM at the respective time point. This may be explained by more aggressive tumors shedding more DNA into the circulation.

Tumor-specific aberrations could be detected in the cfDNA from diagnostic BM-plasma samples and compared to SNP array results of BM-derived DTCs from the same time point in 3 stage M neuroblastoma patients. From one of these patients, SNP array results were also obtained from cfDNA extracted from PB-plasma at the same time point and from tumor tissue which was resected 5 months after diagnosis. The majority of detected genomic aberrations were concordant between the different samples from the respective patient. Interestingly, the BM-plasma DNA and BM-DTCs shared the identical aberrations. The results were completely superimposable as demonstrated not only by the identical aberrations but also the identical breakpoints in all 3 patients. Interestingly, the aberrations detected in the two BM-derived samples from the third patient were not completely concordant with those detected in the PB-plasma, although all samples were collected at the same time point. Notably, there was a higher overlap concerning the detected aberrations between the ctDNA from PB-plasma and

the resected tumor tissue than between the BM-derived samples and the tumor tissue. The aberrations present in the BM-DTCs as well as in the ctDNA from BM-plasma that were not detected in the PB-plasma at the same time point contradict to the current view that cell-free DNA fragments from multiple lesions in the same individual all mix together in the peripheral blood [44, 49]. The findings suggest that the blood-bone marrow barrier may impede the passage of DNA fragments which was also stated for the blood-brain barrier [50]. All in all, these data add another layer of complexity to the phenomenon of tumor heterogeneity as they imply that plasma DNA received from the BM differs from the DNA derived from other tumor cell sources. The detectability of tumor-specific aberrations in the BM- and PB-plasma is dependent on the BM infiltration rate while the aberrations detected in the PB-plasma are not identical to those in the BM-plasma and BM-DTCs.

Our work on stage M neuroblastoma patients shows that plasma-derived ctDNA may serve as an additional source for tumor genome analysis, especially in cases in which bioptic or surgical interventions carry a high risk for the patient. Further comparison of datasets obtained from different intra-patient location will increase the detectability of tumor heterogeneity and thus the understanding of tumor dynamics and progression.

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Supplementary Material

Supplementary Table S1: Comparison of two different cfDNA isolation methods

sample	DNA in plasma (ng/ml)		fold change
	Invitrogen Kit	Qiagen Kit	
1	63.6	26.7	2.38
2	54.0	14.7	3.67
3	59.4	25.5	2.33
4	25.8	46.2	0.56
5	23.0	13.4	1.72
6	136.8	64.2	2.13
7	1314.0	286.2	4.59
8	144.0	47.4	3.04
9	265.8	103.8	2.56
10	205.8	104.4	1.97
		average	2.50

Supplementary Table S2: numerical and segmental chromosomal aberrations including location of breakpoints detected in BM-plasma cftDNA and BM-derived DTCs of patient depicted in Fig. M3A.

Chr., Chromosome; NCAs, numerical chromosomal aberrations; SCAs, segmental chromosomal aberrations; BPs, breakpoints; hmz, region of homozygosity

Chr.	aberration	NCAs	SCAs	BPs
1	pterp21.2(pter-102.1)x2 hmz, p21.2qter(102.1-qter)x3	-	2	1
2	pterqter x3	1	-	-
3	q12.1qter(99.7-qter)x3	-	1	1
4	-	-	-	-
5	pterp15.33(pter-113.6)x3	-	1	1
6	-	-	-	-
7	pterqter x4	1	-	-
8	-	-	-	-
9	pterp21.3(pter-22.0)x1, p21.3p21.2(22.0-27.6)x0, p21.2p21.1(27.6-31.5)x1	-	3	3
10	-	-	-	-
11	q14.2qter(86.2-qter)x1	-	1	1
12	pterp13.31(pter-9.5)x4, p13.31p13.1(9.5-13.5)x2~3, p13.1q24.11(13.5-111.3)x4, q24.11qter(111.3-qter)x4~5	-	4	3
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	pterqter x3	1	-	-
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	pterqter x3	1	-	-
22	pterqter x2 hmz	1	-	-
X	pterqter x3	1	-	-
$\Sigma =$		6	12	10

Supplementary Table S3: numerical and segmental chromosomal aberrations including location of breakpoints detected in BM-plasma cftDNA and BM-derived DTCs of patient depicted in Fig. M3B.

Abbreviations: Chr., Chromosome; NCAs, numerical chromosomal aberrations; SCAs, segmental chromosomal aberrations; BPs, breakpoints

Chr.	aberrations	NCAs	SCAs	BPs
1	-	-	-	-
2	pterp14(pter-65.3)x3	-	1	1
3	pterp25.3(pter-9.5)x1~2	-	1	1
4	q31.1qter(140.1-qter)x1~2	-	1	1
5	pterp15.33(pter-3.3)x1	-	1	1
6	-	-	-	-
7	pterqter x3	1	-	-
8	-	-	-	-
9	pterp13.3(pter-36.2)x1	-	1	1
10	q26.2qter(128.4-qter)x1	-	1	1
11	q12.1q13.4(56.4-71.3)x3, q13.4qter(71.5-qter)x1	-	2	2
12	qterpter x3	1	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	-	-	-
17	q12(32.2-34.2)x3~4, q12qter(34.2-qter)x4~5	-	2	2
18	pterqter x3	1	-	-
19	q13.2qter(42.1-qter)x3	-	1	1
20	-	-	-	-
21	-	-	-	-
22	q11.1q12.2(16.9-31.0)x2~3	-	1	2
X	-	-	-	-
Y	-	-	-	-
$\Sigma =$		3	12	13

Supplementary Table S4: numerical and segmental chromosomal aberrations including location of breakpoints detected in 4 different samples obtained from 1 patient, see Circos plot Fig. M4A.

abbreviations: Chr., Chromosome; BM-DTCs, bone marrow-derived disseminated tumor cells; PB, peripheral blood; TU, tumor tissue;

NCA, numerical chromosomal aberrations; SCAs, segmental chromosomal aberrations; BPs, breakpoints; hmz, region of homozygosity

Chr.	aberrations	BM-DTCs	BM-plasma cftDNA	PB plasma cftDNA	TU after chemo
1	pterp36.11(pter-28.0)x1	✓	✓	✓	✓
	p36.11p34.3(28.0-37.1)x3	✓	✓	✓	✓
	q21.3qter(153.1-qter)x3	✓	✓	✓	-
	q24.1qter(165.9-qter)x3	-	-	-	✓
2	pterp21(pter-42.8)x4	✓	✓	✓	-
	pterp25.1(pter-11.5)x3	-	-	-	✓
	p25.1p21(11.5-42.8)x4	-	-	-	✓
3	pterp21.31(pter-50.2)x1	✓	✓	✓	✓
4	-	-	-	-	-
5	pterp15.33(pter-1.2)x1	✓	✓	✓	-
	pterp15.33(pter-1.2)x3	-	-	-	✓
	q11.2(51.1-56.3)x3	✓	✓	✓	-
	q31.2q34(137.5-167.7)x3	✓	✓	✓	-
	q34qter(167.7-qter)x4	✓	✓	✓	-
	q34qter(167.7-qter)x3	-	-	-	✓
6	pterqter x3	✓	✓	-	-
	pterq26(pter-164.3)x3	-	-	✓	-
	pterp22.3(pter-22.6)x2 hmz	-	-	-	✓
	p22.3q26(22.6-164.3)x3	-	-	-	✓
	q26qter(164.3-qter)x4	-	-	✓	✓
7	pterqter x4	✓	✓	✓	✓
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	pterqter x3	-	-	-	✓
12	q14.2qter(63.6-qter)x3	-	-	-	✓
	q23.3qter(108.5-qter)x3	-	-	✓	-
13	pterqter x3	✓	✓	✓	-
	pterq14.3(pter-54.6)x3	-	-	-	✓
	q14.3qter(54.6-qter)x4	-	-	-	✓
14	-	-	-	-	-
15	-	-	-	-	-
16	q23.3qter(83.3-qter)x1	✓	✓	✓	✓
17	pterp11.2(pter-18.9)x2 hmz	-	-	✓	-
	pterq22(pter-54.0)x3	-	-	-	✓
	p13.1p11.2(7.4-18.9)x1	✓	✓	-	-
	p11.2q22(18.9-54.0)x3	✓	✓	✓	-
	q22qter(54.0-qter)x4	✓	✓	✓	✓
18	pterqter x3	✓	✓	✓	-
	pterqter x4	-	-	-	✓
19	pterp13.3(pter-3.9)x1	✓	✓	✓	✓
	q13.42qter(54.9-qter)x1	-	-	-	✓
20	pterqter x3	✓	✓	-	-
	pterp13(pter-1.7)x1	-	-	-	✓
21	-	-	-	-	-

22	q11.21(20.2-22.0)x7~10 q12.1qter(27.0-qter)x1	✓ ✓	✓ ✓	- ✓	- ✓
X	-	-	-	-	-
Y	-	-	-	-	-
NCA s		5	5	3	3
SCA s		16	16	18	21
BP s		18	18	17	18



Chapter 3

Discussion & Conclusion

3.1. Discussion

The importance of assessing numerical and segmental chromosomal alterations of neuroblastoma cells has become evident when the correlation between such alterations and the tumoral behavior was revealed. The fact that the genetic characteristics of the tumor cells have a large impact on the tumor's aggressiveness highlights the importance of tumor genome analysis at diagnosis in order to determine the patient's prognosis and to identify the optimal therapeutic strategy. The different treatment strategies for neuroblastoma patients range from observation only to surgery and high-dose chemotherapy [2].

Although the genetic analysis of tumor tissue is important at diagnosis, some problems exist: From time to time, bioptic material is not available, for example in case of surgically inaccessible location of the tumor. Taking a biopsy is certainly always related to a risk for the patient and possible clinical complications. In addition to that, commonly performed sample preservation techniques such as the preservation of tumor tissue in formalin-fixed paraffin-embedded (FFPE) blocks may lead to low quality results from molecular analyses due to the fact that the preservation procedure yields cross-linking of DNA. This may especially constitute a problem when working with genome-wide approaches [57].

The probably most prominent hurdle in the analysis of bioptic tumor material is represented by intratumoral heterogeneity. This phenomenon was reported in a variety of studies in different cancer types [103] and was also studied by different groups in neuroblastoma, whereby heterogeneity of *MYCN*, 1p or 11q deletion was observed [126-128].

Liquid biopsies harbor the potential to overcome the mentioned challenges. While the procedure of blood sampling is non- or minimally-invasive and therefore not as inconvenient for the patient as a tumor biopsy, the risk for clinical complications is much lower compared to a surgical intervention. Furthermore, repeated sampling is feasible allowing to monitor the tumor aberrations during the course of disease of neuroblastoma patients.

Concerning the published data on ctDNA in neuroblastoma, only specific loci were so far analyzed in plasma and serum. Due to the importance of genome-wide approaches to analyze the tumor genome, and the fact, that no such approach was performed in neuroblastoma patients up to now, the major aim of this study was to investigate the feasibility of performing tumor genome analysis from plasma samples of neuroblastoma patients.

A more frequent detectability of tumor-specific DNA-fragments in the PB of advanced-stage cancer patients compared to patients with lower stages of disease was shown in a variety of tumor entities [79]. The same effect was also highlighted in a study on neuroblastoma patients when elevated levels of *MYCN* were more reliably detected in the blood of stage 3, 4 and 4S

patients compared to stage 1 and 2 patients [121]. On the basis of these findings, the patient cohort in the study on hand was restricted to stage M (former stage 4) neuroblastoma patients. Besides PB plasma samples, also BM plasma samples were analyzed due to the availability of left-over samples from daily routine blood and bone marrow tests.

In the first part of this work, the amounts of cell-free DNA, i.e. both normal- and tumor-cell derived DNA, per ml of plasma were analyzed in 52 peripheral blood plasma samples and 71 bone marrow plasma samples of 22 stage M neuroblastoma patients. In concordance with published data on the concentration levels of DNA in the circulation of cancer patients [28], the amounts of DNA in the plasma were significantly higher in the neuroblastoma patients compared to 4 control samples from healthy individuals. Furthermore, the DNA-concentrations were significantly higher in bone marrow plasma samples compared to peripheral blood plasma samples, although a large variation was observed in both groups. The latter finding cannot be checked for concordance with other studies due to a lack in published data on DNA from bone marrow plasma.

In this study, the feasibility of assessing the tumor genome from circulating DNA was tested. Thereby, it was determined whether tumor-specific NCAs and SCAs can be identified in DNA extracted from 1 ml of PB- and BM-plasma samples. The identification of such alterations has to be carried out via genome-wide approaches such as aCGH, SNP array analysis or next generation sequencing techniques in order to examine all major genetic modifications of the tumor DNA. These methods differ significantly in their sensitivities. Although sequencing approaches offer high sensitivity and the identification of tumor-specific aberrations down to point mutations, the assays are still too costly to be applied in a routine setting. The use of Ultra-High Density SNP array analysis was chosen in this study as an optimal trade-off between sensitivity and cost efficiency. In this assay, 2.6 million markers, consisting of non-polymorphic markers and SNP markers, ensure the reliable assessment of tumor-specific alterations down to the gene level while additionally providing information on LOH events with and without changes in copy number.

3.1.1. cfDNA-concentrations and sample characteristics

16 samples, consisting of 7 BM-plasma samples and 9 PB-plasma samples were chosen for SNP array analysis. Tumor-specific aberrations were detected in 11 thereof (5 BM- and 6 PB-plasma samples) while in the other 5 cases (2 BM- and 3-PB-plasma samples) “flat profiles” were obtained. The 11 samples with detectable ctDNA proved the feasibility of performing genome-wide analysis of cell-free DNA in neuroblastoma patients in order to analyze the tumor genome. The “flat profiles”, on the other hand, are the result of disomic chromosomes without the presence of any amplifications, gains or losses. These profiles are either a result from the absence of tumor-specific DNA fragments in the plasma samples or, more likely, are due to a minor amount of DNA fragments of tumor-cell origin together with a much larger amount of DNA fragments derived from normal cells. In the latter case, if the fraction of tumor-specific DNA is very low, the aberrations present in the ctDNA are simply “masked” by the normal-cell DNA. The lower detection limit of SNP-array analyses was stated to be less than 5% [129]. However, an immense variation in the amount of ctDNA in the total cell-free DNA fraction was reported to range between 0.01% and 93% [56, 66], thus special techniques are needed to detect low levels of ctDNA.

In order to elucidate under which circumstances tumor-specific aberrations are detectable or not, different characteristics of both the patients and the samples were correlated to the detectability of ctDNA. In a first attempt, a potential relationship to the concentration of cell-free DNA in the plasma samples was investigated in order to determine if a large amount of DNA being present in the plasma is an inevitable consequence of a high amount of cfDNA fragments being shed into the circulation from the tumor. This hypothesis did not hold true as there were samples with low concentrations of cfDNA in which tumor-specific aberrations could be detected by SNP array analysis (e.g. sample #11, see table M1) whereas in other cases, samples with large amounts of cfDNA in the plasma resulted in a flat profile (e.g. samples #3 and #14, see table M1). This observation highlights the differences in the amount of cfDNA between individual patients which seems not only to apply to the tumor-specific fraction of cell-free DNA but also to the normal-cell DNA. It would further be of great interest to learn, whether patients with high levels of cell-free DNA but low fraction of tumor-specific DNA suffered from certain clinical conditions such as fever or inflammation, which could – as previously reported - explain high levels of cfDNA [80].

Due to the fact that DNA was always extracted from 1 ml of plasma, eluted in 30µl of Elution Buffer and 5 µl thereof were used as input for the SNP array analysis, the total amount of input DNA varied between the different samples. However, no correlation between DNA input and detectability of genomic aberrations in the SNP array analysis was found.

In contrast to that, when the BM-infiltration rate, i.e. the percentage of tumor cells in the BM, was compared to the samples with detectable tumor-specific aberrations and the group of samples with “flat profiles”, a significant correlation between an infiltration rate >7.5% and the presence of genomic aberrations was found. This could be explained by more aggressive tumors, i.e. tumors with a higher metastatic potential and therefore more tumor cells in the bone marrow, probably shedding more DNA into the circulation.

3.1.2. DNA-Isolation method

The missing standardization of plasma collection and DNA isolation protocols remains a substantial problem in the field of liquid biopsies. The identification of a reproducible DNA isolation method with an optimal yield of high-quality DNA was therefore a crucial part of this study. All experiments were conducted using 1 ml of plasma samples, because it is not possible to routinely assess large volumes of peripheral blood from neuroblastoma patients due to their very young age. The desired approach should lead to a maximum yield of high quality DNA from this sampling volume while being highly reproducible. Two kits designed for the isolation of DNA from plasma/serum samples based on two very distinct principles were compared. The first kit based on the retention of DNA onto a column and subsequent elution, is broadly used in the field of cfDNA-analysis [63, 130]. The second kit which was chosen for the comparison - a rather rarely-used kit - is based on the binding of DNA to magnetic beads. Ten paired BM-plasma samples were used for this comparison which led to striking results: The bead based method yielded significantly higher amounts of output DNA with an average fold change of 2.5. This difference in the obtained amount of cfDNA can be crucial for downstream analysis and can determine if certain analysis is feasible or not. Based on this result, it is unclear why the bead-based extraction technique has not attracted more attention in the scientific community. Based on our data, it was chosen for all experiments on plasma samples.

3.1.3. Tumor genome analysis

Besides investigating the feasibility of tumor genome analyses from as little as 1 ml of plasma samples in stage M neuroblastoma patients - which could be shown for both PB-plasma samples and BM-plasma samples - a further important aim was to compare the aberrations detected by liquid biopsies to tumor genome analysis data from other tumor cell sources of the respective patient.

As previously reported, disseminated tumor cells in the BM represent an excellent source for tumor genome analyses in neuroblastoma patients [131]. In three cases, in which tumor-specific aberrations were revealed in the analysis of cell-free DNA from BM-plasma samples, SNP array analysis data was also available from the BM-DTCs of the same time point and could be used for comparison of the detected alterations.

In all three cases, completely identical aberrations (gains and losses) with exactly the same breakpoints were identified. The complete congruence between the three sample pairs in this small patient cohort proposes that not only the tumor cells in the BM can be used for tumor genome analysis, but also the cell-free DNA present in the BM plasma can lead to clear results.

Although tumor tissue is still the gold standard for tumor genome analyses, there was only one patient in this study, from whom data from cell-free DNA could be compared to SNP array data of a tissue biopsy. Due to the fact, that this tumor piece was resected after the patient received chemotherapeutic treatment (five months after the time point of diagnosis), this comparison of data had to occur under the consideration that the sampling of the different specimens happened at different time points within the patient's course of disease. In addition to the tumor biopsy, three samples from this patient were obtained at the time point of diagnosis: a bone marrow sample with an infiltration rate of 40% as well as two plasma samples, one from the BM-plasma and one from the PB-plasma. It is important to emphasize, that all three samples were obtained at the same time point, i.e. at diagnosis. When the four different samples from this patient were compared, we found that the majority of numerical as well as segmental aberrations were shared between all samples. The tumor tissue sample, that was obtained five months after the other three samples, differed strongly from the other specimens concerning the detected aberrations as well as the location of the respective breakpoints. This can be explained by the time dependent tumor evolution and/or treatment induced genomic changes.

Comparing the three diagnostic samples, i.e. BM-DTCs, cell-free DNA from BM-plasma and cell-free DNA from PB-plasma, revealed surprising results: While the genomic changes of BM-DTCs were superimposable with the cfDNA obtained from the BM-plasma, differences between the two sources of cfDNA, PB and BM, from the same time point were noticed. While 15 breakpoints were shared across all three diagnostic samples, one single breakpoint was detected in the PB-plasma only and three breakpoints were present in two bone marrow samples only. Notably, two of these three breakpoints derived from a large amplicon on the long arm of chromosome 22 with a size of approximately 2 million base pairs and maximum copy number values of around 10. This amplicon is present in the two BM-derived samples only and neither in the cell-free DNA of the PB-plasma nor in the tumor tissue. In addition to that, one breakpoint on the same chromosome arm is shared between all four analyzed samples.

To find this amplicon in the BM samples is striking in different aspects: It contradicts the currently wide-accepted view that the same cross-section of the tumor genome is present in all

bodily fluids. It was stated by different groups that plasma analysis can overcome different forms of molecular heterogeneity because fragments derived from all tumor sources in a patient's body are present together in the bodily fluids [57, 111]. However, to the best of our knowledge, no comparison of PB-plasma and BM-derived plasma was done so far. The blood-brain barrier was reported as a potential hurdle that prevents DNA fragments released from brain-tumors to enter the blood stream [79]. The findings presented in this work suggest a similar role for the peripheral blood-bone marrow barrier.

3.1.4. Liquid biopsies – unresolved issues

Despite the manifold potential utilities of liquid biopsies, some limitations and open questions remain. In studies comparing the ctDNA levels from plasma or serum between patients with different stages of cancer, the fraction of patients with detectable levels of ctDNA was rather small in patients with low stages of disease. For example, as aforementioned, the level of ctDNA was stage-dependent ranging from 27% to 82% in stages 1 to 4 in a large study including over 600 patients with various cancer types [79]. In another study applying a highly sensitive targeted sequencing approach in plasma samples from lung cancer patients, ctDNA could be detected in 100% of analyzed patients with stage 2-4 disease but only in 50% of patients with stage 1 cancer [78]. The same trend was also observed in neuroblastoma patients when differences in the detectability of MNA in plasma samples were stated between patients with low stage of disease compared to patients with higher stages [121]. These findings led to the decision to include stage M neuroblastoma patients only in the study on hand.

Cell-free DNA is present in the plasma of all humans [132] due to cell death occurring under normal conditions as well as during different types of disease. This ubiquitously occurring cell death leads to release of DNA into the circulation from various cell types [28]. In plasma/serum samples of cancer patients, in which only a minor fraction of cfDNA is tumor-derived, the mutated sequences and tumor-specific aberrations can therefore be “masked” by a large fraction of normal cell-derived DNA. The sensitivity and specificity of the detection system is thus extremely important to detect also minor amounts of ctDNA.

It is still under debate whether plasma or serum is better suited for ctDNA experiments. For this study, plasma samples were chosen over serum samples, because several groups showed that the amount of cfDNA extracted from serum samples is higher compared to plasma samples, which is most likely due to a high amount of cfDNA being released from destruction of white blood cells during the clotting process [75, 133, 134]. Plasma DNA may therefore better resemble *in vivo* conditions than serum samples [133, 135]. Furthermore, Jung et al. showed that the concentration of DNA in plasma samples was more stable during storage at room temperature compared to serum samples [134]. Taken together, to reduce the risk of detecting

large amounts of normal cfDNA masking the tumor-derived DNA, as well as the higher stability of cfDNA in plasma samples, led to the decision to only include plasma samples in the study on hand.

Lastly, missing methodological standardization is a main issue in the field of ctDNA analysis, that possibly renders results non-reproducible and limits the comparability of data between different studies [54]. Different groups have tried to identify the optimal method for the collection of plasma from whole-blood samples [136-138] as well as the isolation of cfDNA from plasma samples [139-142]. Due to the contradictory outcomes of those studies, the identification of a robust method for the isolation of DNA from plasma samples was a crucial aspect in the presented work.

3.2. Conclusion

In this study, the feasibility of genome-wide analyses of cell-free DNA from peripheral blood plasma samples as well as bone marrow plasma samples was shown. Although the concentrations of DNA in the cell-free PB compartment show a large variation between different patients, pan-genomic analyses were shown to reveal tumor-specific aberrations in samples from stage M neuroblastoma patients with a bone marrow involvement over 7.5 %.

In contrast to the correlation of ctDNA and bone marrow involvement, the detectability of tumor-specific DNA did not depend on the total amount of DNA per ml of plasma or the input amount of DNA into the SNP array analysis.

The comparison of data sets between cell-free DNA from peripheral blood- as well as bone marrow-plasma samples revealed that aberrations found in bone marrow-derived disseminated tumor cells as well as cell-free DNA from the bone marrow plasma were completely identical in three patients while in one patient, differences were observed between the two bone marrow derived samples and cell-free DNA from the peripheral blood plasma although all three samples were collected at the same time point. Notably, the tumor tissue of this patient, which was analyzed five months later, showed high concordance with the three samples while also harboring several different aberrations. It is a noteworthy contradiction that although the detectability of tumor-specific DNA fragments in the blood-plasma depends, as aforementioned, on the infiltration rate of tumor cells in the BM, the amplicon present in the bone marrow is not detectable in the PB-plasma.

Due to the rather small patient cohort, further investigations are necessary to confirm these findings. By using a more sensitive genome-wide approach, tumor-specific aberrations may also be detectable in samples in which the fraction of tumor-derived DNA fragments was too

low for identification with SNP array analysis. The concordance between bone marrow plasma DNA and DTCs in the bone marrow has to be validated in a larger patient cohort. Furthermore, it will be necessary to compare cell-free DNA from peripheral blood and bone marrow samples to determine whether differences are frequently observed. If so, it has to be investigated, which of the samples better resembles the diagnostic tumor. In large prospective studies, it should be investigated, if the relapse clone can be detected in the plasma at diagnosis. Further and more comprehensive studies on cell-free DNA could therefore lead to a deeper understanding of the tumor evolution during the course of disease.



Chapter 4

Appendix

List of Abbreviations

aCGH	array comparative genomic hybridization
BM	bone marrow
BP	breakpoint
CA	cancer antigen
CEA	carcino-embryonic antigen
cfDNA	cell-freeDNA
Chr	Chromosome
cnLOH	copy-neutral Loss of Heterozygosity
CRC	colorectal cancer
CT	computed tomography
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
DTCs	disseminated tumor cells
EGFR	epidermal growth factor receptor
FFPE	formalin-fixed paraffin-embedded
INRG	International Neuroblastoma Risk Group
INSS	International Neuroblastoma Staging System
LOH	Loss of Heterozygosity
MNA	<i>MYCN</i> amplification
MRD	minimal residual disease

NCA	numerical chromosomal aberration
NGS	next generation sequencing
NSCLC	non-small-cell lung cancer
PB	peripheral blood
PCR	polymerase chain reaction
PET	positron emission tomography
PSA	prostate-specific antigen
SCA	segmental chromosomal aberration
SLE	systemic lupus erythematosus

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Abstract

Tumor genome analysis is crucial in neuroblastoma patients but frequently limited by unavailable bioptic material and intratumoral heterogeneity. Tumor genotyping is one of the main suggested clinical applications of circulating tumor DNA (ctDNA) in addition to monitoring of treatment, and early detection of recurrence. ctDNA refers to the tumor-derived fraction of circulating cell-free DNA (cfDNA) and is well described in the plasma of cancer patients, especially of those with metastatic disease.

Due to a lack in published data on genome-wide analyses of ctDNA in pediatric cancer patients, in this study, the feasibility of performing SNP array analysis from 1 ml cell-free peripheral blood (PB)- or bone marrow (BM)-plasma in order to identify tumor-specific genomic aberrations was investigated.

Cell-free DNA was obtained from 52 PB- and 71 BM-plasma samples of 22 stage M neuroblastoma patients. cfDNA was extracted from the plasma using a magnetic bead enrichment technique that yielded higher levels of DNA as compared to a conventional extraction method.

Tumor-specific aberrations were detected in 11 out of 16 samples by SNP array analysis. The feasibility of retrieving tumor-specific genomic aberrations from 1 ml of cell-free plasma DNA was thereby shown for both PB- as well as BM-derived samples.

The detectability of tumor-specific DNA fragments did neither depend on the amount of cell-free DNA per ml of plasma nor on the DNA input for the SNP array analysis, but rather on the bone marrow infiltration rate.

We compared different SNP array results from single patients obtained from different tissues/sources, for example tumor tissue with plasma ctDNA, or BM-derived disseminated tumor cells (DTCs) with ctDNA from PB-plasma and ctDNA from BM-plasma. In the analyzed patient cohort, the majority of detected genomic aberrations were concordant between the different samples from the same patient. However, while ctDNAs from BM-plasma and BM-DTCs showed the identical genomic aberrations in three patients, ctDNAs from BM- and PB-plasma were not superimposable concerning their alterations in one patient.

Based on the data presented, plasma-derived ctDNA may serve as an excellent source for tumor genome analysis, especially in cases in which bioptic or surgical interventions carry a high risk for the patient. The findings contradict the current view that all bodily fluids harbor the identical tumor genome. Therefore, the comparison of intra-patient data sets is needed to obtain a better understanding of tumor heterogeneity and of the dynamics of the tumor within the course of disease.

Kurzfassung

Die genetische Heterogenität innerhalb des Tumors sowie oftmals nicht verfügbares Biopsiematerial limitieren die genaue Analyse des Tumorgenoms, welche speziell beim Neuroblastom von großer Bedeutung ist. Die Genotypisierung des Tumors ist, neben dem Therapie-Monitoring und der Früherkennung von Tumorrezidiven und minimalen Resterkrankungen, eine der wichtigsten potentiellen Anwedungen der Analyse von zirkulierender Tumor DNA. Das Vorkommen von zirkulierender Tumor DNA, welche den Tumor-spezifischen Anteil der zell-freien DNA darstellt, wurde im Plasma von Krebspatienten, vor allem von jenen mit einem metastasierten Krankheitsbild, von einer Reihe von Autoren umfassend beschrieben.

Da bisher keine Daten von genom-weiten Analysen der zirkulierenden Tumor DNA im pädiatrischen Bereich publiziert wurden, sollte in dieser Studie getestet werden, ob zell-freie DNA von 1 ml Plasma mittels SNP-Array Analyse untersucht werden kann, und ob Tumorgenom-spezifische Aberrationen identifizierbar sind.

Von 22 Stadium M Neuroblastom-Patienten wurden 123 Plasmaproben untersucht, 52 waren aus dem peripheren Blut (PB) und 71 aus dem Knochenmark (KM). Eine auf Magnetic Beads basierende Methode, die sich im durchgeführten direkten Vergleich zu einer herkömmlichen Extraktionsmethode in Bezug auf die Output-Menge an DNA als überlegen herausgestellt hat, wurde verwendet um zell-freie DNA aus den Plasmaproben zu isolieren.

Mittels SNP-Array Analyse konnten Tumor-spezifische Aberrationen in 11 von 16 analysierten Proben nachgewiesen werden. Die Möglichkeit, genomische Aberrationen des Tumors in 1 ml zell-freien Plasma zu detektieren, konnte dabei sowohl für PB- als auch KM-Plasmaproben gezeigt werden.

Die Detektierbarkeit von Tumor-spezifischen DNA-Fragmenten war weder von der DNA-Konzentration im Plasma noch von der DNA-Input Menge für die Analyse abhängig, sondern vielmehr von der Tumorzell-Infiltrationsrate im KM.

Die Resultate der SNP-Array Analysen wurden mit Daten von anderen Geweben bzw. anderen Körperflüssigkeiten desselben Patienten verglichen. In dem dabei analysierten Patientenkollektiv wurde eine hohe Übereinstimmung der genomischen Aberrationen zwischen den untersuchten Geweben bzw. Plasmaproben festgestellt. Doch während die zell-freie DNA von KM-Plasmaproben mit den disseminierten Tumorzellen des KM in allen 3 Fällen eine 100%ige Übereinstimmung aufwies, waren die Aberrationen zwischen den zell-freien DNAs von PB- und KM-Plasmaproben in einem weiteren Fall nicht komplett deckungsgleich.

Die genom-weite Analyse von zirkulierender Tumor DNA aus Plasmaproben birgt hohes Potential, vor allem in Fällen, in denen eine Biopsie ein hohes Risiko für den Patienten darstellt.

Die Erkenntnisse dieser Studie stehen im Widerspruch zu der gegenwärtigen Ansicht, dass die identen Genomveränderungen in allen Körperflüssigkeiten vorhanden sind. Ein systematischer Vergleich verschiedener Datensätze innerhalb einzelner Patienten wäre notwendig um ein besseres Verständnis der Tumorheterogenität sowie der Tumorevolution im Krankheitsverlauf zu erlangen.

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	Assoc. Prof. Peter F. Ambros, PhD „Performance and Analysis of Ultra-High Density SNP Arrays in Neuroblastoma Patients“
03/2014	Internship at the Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria Ass. Prof. Gerda Egger, PhD „In vitro knock out of the DNA methyltransferase DNMT1 in mouse T cell lymphoma cell lines“
04/2012 – 02/2013 09/2013	Internship and further employment in Molecular Diagnostics Department of Health & Environment AIT – Austrian Institute of Technology, Vienna, Austria Assoc. Prof. Andreas Weinhäusel, PhD Christa Nöhammer, PhD
03/2012	Internship Microbiology and Bacterial Diagnostics Labcon Medizinische Laboratorien GmbH Krankenhaus Göttlicher Heiland, Vienna, Austria Univ.Do. Dr.med. Stylianos Kapiotis,
09/2011 – 11/2011	Bachelor Thesis at the Institute for Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Marie-Theres Hauser „Überprüfung von Proteininteraktionen der ESCRT-III Komponente VPS2.2 mithilfe des „yeast-two-hybrid“- Systems“
07/2010 – 08/2010	Internship at the Department of Clinical Pathology, Microbiology and Infectious Diagnostics

Krankenhaus der Barmherzigen Schwestern, Ried im
Innkreis, Austria

Prim. Dr. Gabriele Brinninger

Scientific Presentation

02/2015

The Molecular Medicine Tri Conference 2015

San Francisco, California, USA

Poster Presentation: „Genome-wide SNParray analysis
reveals tumor specific aberrations in the plasma DNA of
pediatric cancer patients“

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Date

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Signature