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RESEARCH ARTICLE

Cancer therapy monitoring in xenografts by quantitative analysis of circulating tumor DNA

Tobias M. Gorges, Johanna Schiller, Arndt Schmitz, Daniel Schuetzmann, Christoph Schatz, Thomas M. Zollner, Thomas Krahn, and Oliver von Ahsen

Global Biomarker Research, Bayer Pharma AG, Berlin, Germany

Abstract

Context: Circulating tumor DNA (ctDNA) is a promising biomarker in cancer.

Materials and methods: We generated xenograft models of cancer and detected ctDNA in plasma by qRCR targeting human AluJ sequences.

Results: Our assay reached single cell sensitivity *in vitro* and a correlation between ctDNA amount and tumor size was observed *in vivo*. Treatment with a mitogen activated protein kinase kinase (MEK)-inhibitor (BAY 869766) reduced ctDNA levels. Using this assay, we also confirmed that high levels of cell-free DNA are found in cancer patients compared to healthy individuals.

Discussion and conclusion: We show that ctDNA may be useful biomarker for monitoring tumor growth and treatment response.

Keywords: ctDNA, biomarker, AluJ, xenograft, BAY 869766

Introduction

The presence of cell-free DNA (cfDNA) in human plasma and serum was first reported by Mendel and Métais in 1948 (Mendel & Métais 1948). The source of cfDNA in blood, urine, stool, milk, bronchial lavage, or ascites fluid has not been clearly elucidated yet. Two theories on the origin of cfDNA are described: Either the DNA fragments enter the bloodstream following cell death by apoptosis and necrosis (Jahr et al. 2001), or they are actively released by living cells (van der Vaart & Pretorius 2008). The fact that highest sensitivity and discrimination between tumor and nontumor DNA could be achieved by testing small amplicons, argues for an apoptotic origin of the DNA in concordance with the nucleosomal DNA fragmentation pattern (the classical "DNA laddering") leading to overrepresentation of smaller fragments (Mouliere et al. 2011).

In 1977, Leon et al. found elevated levels of cfDNA in cancer patients compared to healthy controls and associated poor survival with high DNA levels in serum (Leon et al. 1977). Several studies have investigated the relevance of cfDNA in clinical studies showing a correlation between the amount of cfDNA and disease progression or prognosis (Gautschi et al. 2004; Catarino et al. 2008; Sunami et al. 2008; Schwarzenbach et al. 2008; van der Drift et al. 2010). However, there are also studies in which quantification of cfDNA could not discriminate cancer patients from other individuals. Schmidt et al. (2008) could not find significant differences in levels of cfDNA purified from plasma, serum, and bronchial lavages in lung cancer patients compared to patients with benign lung diseases. High levels of cfDNA in plasma have also been described for benign gastrointestinal disease (Shapiro et al. 1983) and for chronic inflammatory diseases (Koffler et al. 1973; Leon et al. 1977). Although divergent results are published, levels of cfDNA in an individual patient still might be a useful marker to assess response to treatment or to detect a relapse, provided that

Tobias M. Gorges and Johanna Schiller contributed equally to the work.

Address for Correspondence: Oliver von Ahsen, Boehringer Ingelheim Pharma GmbH & Co. KG, DMPK Biomarkers, Biberach, Germany. Tel: +49 (7351) 54-5543. Fax: +49 (7351) 83-5543. Email: Oliver.von_ahsen@boehringer-ingelheim.com

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the cfDNA is indeed tumor derived and not originating from damaged surrounding tissue (Schwarzenbach et al. 2008, Zitt et al. 2008). The best approach to discriminate between tumor and nontumor DNA is to specifically detect tumor-associated mutations. Although specific mutations have to be detected for every patient, this strategy allows for background free tumor growth and therapy monitoring (Diehl et al. 2008). The use of preclinical animal models could help to understand the relationship between levels of cfDNA and cancer disease in a much defined setting, where human-specific sequences are tumor derived (ctDNA) by definition.

To our knowledge, few publications describe the detection of ctDNA in mouse models. Tumor burden could be monitored in xenografts by the detection of *hLINE-1* sequences (*human long interspersed nuclear elements-1*) (Rago et al. 2007). Just recently, Thierry et al. (2010) could specifically detect human-specific ctDNA in xenografts and discriminated it to murine nontumor DNA, showing increased ctDNA levels with larger tumors. In addition, García-Olmo et al. (2006, 2008) published a number of articles measuring ctDNA levels in a syngenic rat model, in which tumor-derived ctDNA was discriminated from host cfDNA by detection of a specific *KRAS* mutation (Samos et al. 2006). However, only one study addressed changes in response to an anticancer agent (Kamat et al. 2006).

We established a broadly applicable method for highly sensitive detection of ctDNA to investigate ctDNA kinetics under treatment using a novel MEK-inhibitor (BAY 869766) (Iverson et al. 2009).

We analyzed the kinetics of ctDNA in animal models amplifying human *AluJ* sequences which have been used before to test the metastatic spread into secondary organs (Schneider et al. 2002). By quantification of *AluJ* sequences, response to therapy could be shown in xenografts. Using this approach, we also tested the levels of cell-free DNA in colorectal cancer patients. As the cfDNA levels in cancer patients are elevated compared to healthy controls, the established assay can now be used in clinical studies to test the response to therapy.

Methods

Cell culture

We used the b-raf, APC, and SMAD4 mutated colorectal cancer cell line Colo205 (ATCC, #CCL-222), the basal-like estrogen receptor (ER) negative breast cancer cell line MDA-MB-231 characterized by k-ras, b-raf, p53, and CDKN2a mutations (ECACC, #92020424) or a subclone of MDA-MB-231 which was isolated from bone marrow metastasis, MDA-MB-231-SA (provided by Dr. Käkönen). The epithelial ER negative breast cancer cell line MDA-MB-468 carrying mutations in RB, PTEN, p53, and SMAD4 (ATCC, #HTB-132) and KPL-4, driven by a Her2 amplification (provided by the Kawasaki Medical School) were used.

All human cancer cell lines were cultured in accordant media together with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO_2. Adherent cells were harvested at 80% confluency by washing with sterile Dulbecco's phosphate buffered saline (PBS; Invitrogen Corporation) followed by trypsinization for 5 min at 37°C.

DNA extraction

DNA extraction was performed using the QIAamp DNA Micro-Kit for longitudinal measurements (15 µL plasma) (Qiagen, Cat. no 56304), or the QIAamp DNA Mini-Kit (Qiagen, Cat. no 51304) after final blood collection (200 µL plasma). Samples were processed as described by protocol of the vendor. DNA concentration was determined with a Nanodrop spectrophotometer and samples were stored at –20°C until use.

Quantitative polymerase chain reaction

SYBR Green based quantitative polymerase chain reaction (qPCR) was used to quantify human DNA extracted from plasma samples. The qPCR assay consisted of QuantiTect SYBR Green PCR Master Mix (Qiagen Cat. no. 204243), 400 nM primers, and 1–5 µL of template DNA. qPCR was performed on ABI 7900HT real-time cycler under the following conditions: an initial denaturation step at 95°C for 15 min followed by 40 cycles of denaturation at 94°C (15 s) and an annealing and elongation step at 60°C for 1 min.

All samples were measured in duplicates or triplicates. Data were analyzed using SDS 2.3 software. Negative samples (H_2O or plasma samples of naive animals) were included in every run. ctDNA concentrations were calculated from a standard row which was included in every run. As sequence size was found to be important when quantifying ctDNA, only short amplicons were used.

Human-specific primers were used for the detection of *GAPDH* (forward 5′-*ATCATCCCTGCCTCTACTGG*-3′; reverse 5′-*GTCAGGTCCACCACTGACAC*-3′) resulting in a 121 base pair amplicon, the multicopy sequences *hLINE-1* (forward 5′-*TCACTCAAAGCCGCTCAACTAC*-3′; reverse 5′-*TCTGCCTTCATTTCGTTATGTACC*-3′) generating an amplicon of 81 base pairs (according to Rago et al. 2007) and *AluJ* (forward 5′-CACCTGTAATCCCAGCACTTT-3′; reverse 5′-CCCAGGCTGGAGTGCAGT-3′) (Schneider et al. 2002) generating an amplicon of 240 base pairs.

Spiking experiments

To test the sensitivity of the assays, 200 µL of plasma were taken from naive mice and spiked with different concentrations of human DNA extracted from the used cancer cell line MDA-MB-231 (0.01 pg to 10 ng/µL) or 1 to 100 cancer cells. DNA of the "spiking sample" was purified using the QIAamp DNA Mini-Kit (Qiagen, Cat. no 51304) and qPCR was performed comparing all three primer sets.

Generation of cancer xenograft models and blood collection

Five-to-six-week-old female NCr nude mice (TACONIC) were used throughout all xenograft studies. All experiments were approved by the local regulatory agency Landesamt für Gesundheit und Soziales Berlin (approval number: 1403). Mice were housed according to institutional guidelines with highest animal welfare standards (Workman et al. 2010). For *in vivo* inoculation, harvested cells were counted by CASY DT Cell Counter and Analyser (Innovatis AG). Cell number and viability were determined by the Analyser Software. Human cancer cells (MDA-MB-231, MDA-MB-468, KPL-4, and Colo205) were harvested by trypsinization, diluted with a mixture of PBS plus Matrigel (1:1) and kept at 4°C for maximum of 1 h. Tumor cells of 1 to 3 × 10^6 per animal were injected either into the mammary fat pads (m.f.p.) or subcutaneously. Tumor size was determined twice a week by external caliper (length × width). For 200 µL of plasma collection mice were sacrificed at the end of each experiment. Prior to blood sampling the animals were anesthetized with Rompun and Ketavet. For longitudinal measurements it was necessary to collect multiple blood samples from the mice (30–40 µL). To reduce stress for the animals, the minimally invasive procedure of blood collection from the lateral tail vein was chosen.

Cancer xenograft models under treatment

A treatment study was performed to test the novel MEK-inhibitor (BAY 869766) (Iverson et al. 2009) in an orthotopic breast cancer model.

The MEK-inhibitor BAY 869766 selectively binds to an allosteric binding site in the MEK1/2 enzymes, blocks their kinase activity and inhibits cell proliferation in several tumor cells *in vitro* with single digit nanomolar potency in b-raf mutated cell lines and double digit nanomolar potency in k-ras mutated cell lines. The inhibitor also potently blocks tumor growth *in vivo*.

1 × 10^6 MDA-MB-231 cells were orthotopically injected into the m.f.p. of female nude mice. When the tumors reached a size of 50 mm^2, mice were randomized into the different treatment groups. All animals were treated daily for 15 days: Vehicle group A received vehicle (30% Hydroxypropyl-b-cyclodextrin in water, 10 mL/kg p.o.) whereas group B was treated with a MEK-inhibitor (3 mg/mL in 30% Hydroxypropyl-b-cyclodextrin in water, 10 mL/kg p.o. corresponding to 30 mg/kg). Prior to therapy, six untreated animals bearing tumors of 50 mm^2 and six naive mice were analyzed to generate positive and negative controls, which were included in every run. For ctDNA-concentration determination 200 µL of plasma were used.

To analyze ctDNA kinetics under treatment by collection of only 15 µL of plasma, a second treatment study was performed. Mice were subcutaneously inoculated with 3 × 10^6 cells of the Colo205 line. When tumors reached a size of 50 mm^2, animals were separated into two groups (n = 4/group) and treatment with the MEK-inhibitor or the vehicle started as described before. Plasma samples were analyzed after 1, 3, 5, and 8 days of treatment.

Clinical samples

Clinical samples were obtained from Indivumed (Hamburg, Germany). All plasma samples originate from patients scheduled for the resection of primary colorectal carcinomas. The plasma samples were taken within the last 48 h before surgery. The tumors were all classified as malign moderately differentiated adenocarcinomas, one case partially mucinous. All tumors were grade 2, the tumor sizes ranged from 3 to 12 cm. The complete clinical data set is available as Supplemental File. Plasma samples out of 10 female and 10 male healthy individuals were collected in house and served as negative controls. DNA was isolated (50 µL plasma) using the QIAamp DNA Mini-Kit (Qiagen, Cat. no 51304). Samples were processed as described by protocol of the vendor, and stored at –20°C until use. SYBR Green based qPCR was used to quantify cfDNA amount. The qPCR assay consisted of QuantiTect SYBR Green PCR Master Mix (Qiagen Cat. no. 204243), 400 nM primers, and 5 µL of template DNA. All samples were measured in triplicates as mentioned before. Negative sample (H_2O) was included in every run. ΔCq values were calculated by: Cq value of the sample subtracted from the Cq value of the negative control (H_2O).

Results

Detection of human DNA – *ex vivo*

In murine xenograft models, human (tumor) DNA can easily be discriminated from host DNA by targeting human-specific sequences. For assay validation three different candidate markers were analyzed (*GAPDH*, *hLINE-1*, and *AluJ*) using either isolated DNA or spiked cells of the MDA-MB-231 cell line. Murine DNA and H_2O samples were used as negative controls (background). Using primers against the single-copy gene *GAPDH*, we could distinguish spiked human DNA with concentrations of 0.01 ng/µL from the background. However, 20 human cells had to be spiked into the sample in order to generate a signal above background. As expected, higher sensitivity was observed targeting multicopy DNA sequences. Highest sensitivity was obtained for human *AluJ* elements (Figure 1A and 1B). Using the multicopy sequences, the assays reached single cell sensitivity (Figure 1B). As highest sensitivity was observed targeting the *AluJ* sequences we wanted to define the limit of detection (LOD) for this assay. In repeated spiking experiments (n = 6), we found that concentrations of ≥0.2 pg/µL could be discriminated from the background targeting the *AluJ* sequences (Figure 1C shows a representative spiking experiment). We measured a Cq value of 24.147 ± 0.055 for 0.2 pg/µL and 25.787 ± 0.070 in the background control. The data ranges are significantly separated by more than three

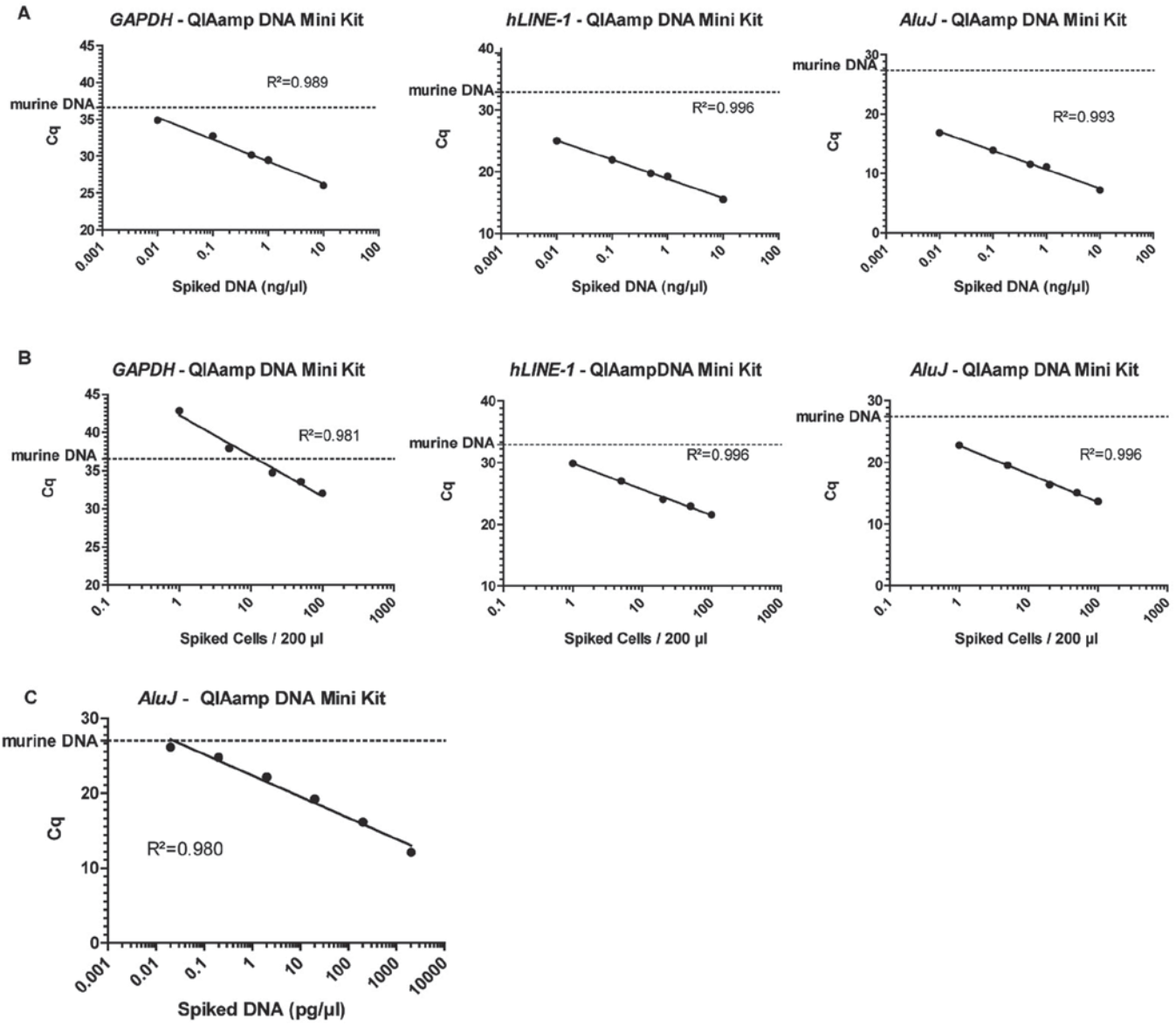

Figure 1. Detection of human DNA in murine plasma. (A) Human DNA concentrations from 0.01 to 10 ng/µL or (B) 1 to 100 human breast cancer cells (MDA-MB-231) were spiked into 200 µL of murine plasma. DNA was isolated and detected by qPCR using primer sets specific for human *GAPDH*, *hLINE-1*, or *AluJ* DNA sequences. Naive murine plasma samples were used as negative controls "murine DNA" (background). (C) Determination of the limit of detection (LOD) targeting the *AluJ* sequences in spiked samples with human DNA concentrations from 0.02 to 2000 pg/µL plasma. Concentrations below 0.2 pg DNA/µL murine plasma were considered as background. Shown graphs are representative for six independent spiking experiments.

standard deviations. The coefficients of variation (CV) in the titration of spiked DNA shown in Figure 1C are all below 2%. Based on our findings, the *AluJ* qPCR assay was chosen for further *in vivo* studies.

Monitoring tumor growth by ctDNA levels

To test whether ctDNA levels differ between subcutaneous and orthotopic models, KPL-4 tumor cells were injected into the right flank (subcutaneously) or into the mammary fat pad (orthotopically) of the animals. ctDNA analysis in 200 µL plasma samples was performed when tumors reached a size of 20, 40, 60, 80, and 110 mm^2, respectively ($n = 5$/group and time point). We could show that ctDNA levels increased with tumor size for both subcutaneous and orthotopic xenografts (Figure 2A and 2B).

ctDNA levels under treatment with a MEK-inhibitor (200 µL plasma)

Having shown that ctDNA levels reflect tumor progression *in vivo*, we subsequently assessed ctDNA kinetics under treatment. For this aim, MDA-MB-231 tumor cells were injected into the m.f.p. of the mice. When tumors reached an average size of 50 mm^2, all animals were treated once daily for 15 days with the MEK-inhibitor (BAY 869766) or vehicle solution. During the course of treatment, quick and significant tumor shrinkage was observed in the therapy group until 8 days of treatment (Figure 3A).

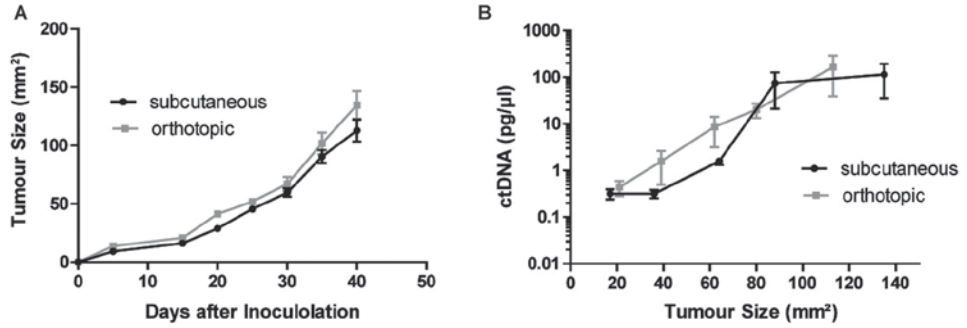

Figure 2. Comparison of tumor growth and ctDNA kinetics in subcutaneous and orthotopic xenograft models. (A) 1×10^6 cells of the KPL-4 line were injected into 50 female nude mice (n = 25/group). Tumor size was assessed twice weekly and tumor area was calculated from measured length and width. Plasma samples (200 µL) were collected when tumors reached a size of 20, 40, 60, 80, and 110 mm² (n = 5/timepoint and group). (B) qPCR targeting human *AluJ* sequences was used to detect ctDNA amount. ctDNA concentrations (pg/µL plasma) were calculated based on spiked standard rows which were included in every run. qPCR measurements were done in duplicates.

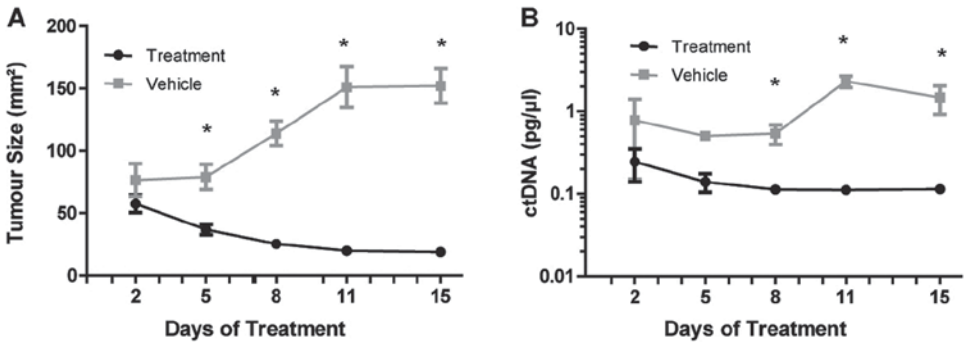

Figure 3. ctDNA kinetics during treatment. (A) 1×10^6 cells of the MDA-MB-231 line were orthotopically injected into 60 female nude mice (n = 30/group). Daily treatment started as tumors reached a size of 50 mm². Group A received the vehicle solution and group B the MEK-inhibitor (BAY 869766). Tumor size was measured at time point of autopsy. (B) ctDNA load in pg/µL of plasma were analyzed 2, 5, 8, 11, and 15 days after start of treatment (n = 6/time point and group). qPCR targeting human *AluJ* sequences was used to detect ctDNA. ctDNA concentrations were calculated based on spiked standard rows that were included in every run. Data were analyzed by unpaired t-test and significant differences ($p < .05$) are indicated by asterisk.

Analyzing kinetics of ctDNA, we found increased ctDNA levels in the vehicle group whereas decreasing ctDNA signals were seen in plasma samples of the treatment group (Figure 3B).

Serial monitoring tumor growth by ctDNA levels using 15 µL of plasma

Having validated the ctDNA measurements for 200 µL of plasma, we next wanted to find out if longitudinal measurements also reflect tumor burden *in vivo*. For this aim, 30–40 µL of blood were collected from the tail veins of tumor bearing animals and the resulting plasma tested for human *AluJ* sequences. As apparent in Figure 4A, the *AluJ* quantification assay was able to detect ctDNA signals after 3 weeks of tumor growth. We could show that the amount of ctDNA increased with growing tumor size in every group. Tumors of the MDA-MB-468 line started to shrink after 46 days which was also reflected by decreased ctDNA levels during the last measurement (Figure 4A).

Our data indicate a strong correlation between tumor size and ctDNA plasma concentration, which becomes especially evident when animals are analyzed individually (Figure 4B–4D). However, the absolute amount of released ctDNA seems to be cell line specific, as animals injected with cells of the MDA-MB-231 line showed lower ctDNA levels when compared to animals of the MDA-MB-468 or KPL-4 group with similar tumor mass.

ctDNA levels under treatment with a MEK-inhibitor (15 µL plasma)

To analyze ctDNA kinetics under treatment using as little as 15 µL of plasma, a second treatment study was performed. Subcutaneous injection of colorectal cancer cells (Colo205) was chosen as tumor size is hard to monitor in orthotopic colorectal xenografts. Treatment with the MEK-inhibitor (BAY 869766) started when tumors had reached an average size of 50 mm². Measurement of tumor size revealed the effect of the compound as the tumors in the treatment arm shrank while the tumors in the vehicle group continued to grow (Figure 5A). Blood samples were collected from the tail vein of individual animals and analyzed for ctDNA. ctDNA concentrations reflected the tumor size, as no notable ctDNA load was found in the plasma of animals in the treated group. In contrast to that, a considerable increase in ctDNA amount was observed in xenografts receiving the vehicle solution when the tumors grow to larger sizes (Figure 5B). Hence, we could also confirm the practicability and significance of serial ctDNA

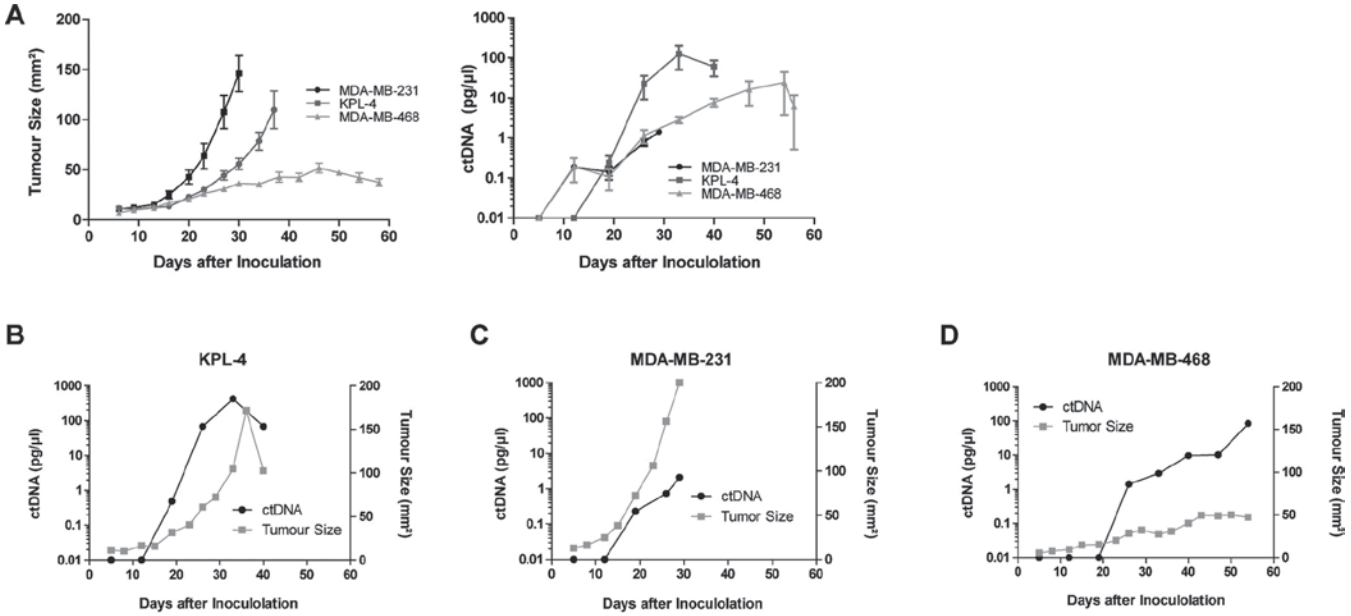

Figure 4. Longitudinal ctDNA measurements. (A) Tumor growth was analyzed in different orthotopic breast cancer xenograft models inoculated with 1×10^6 cells of the MDA-MB-231, MDA-MB-468 or KPL-4 line (n = 7/group). Tumor size was assessed twice weekly. Tumor area was calculated from measured length and width. Blood sampling from the tail vein was done twice weekly for longitudinal ctDNA measurements. qPCR targeting human *AluJ* sequences was used to detect ctDNA and concentrations were calculated based on spiked standard rows. Concentrations are defined as pg/µL plasma. (B–D) To compare tumor growth and plasma ctDNA concentration for each animal individually over the course of the experiment, both variables were plotted against the timeline as x axis. Shown graphs are representative for all samples.

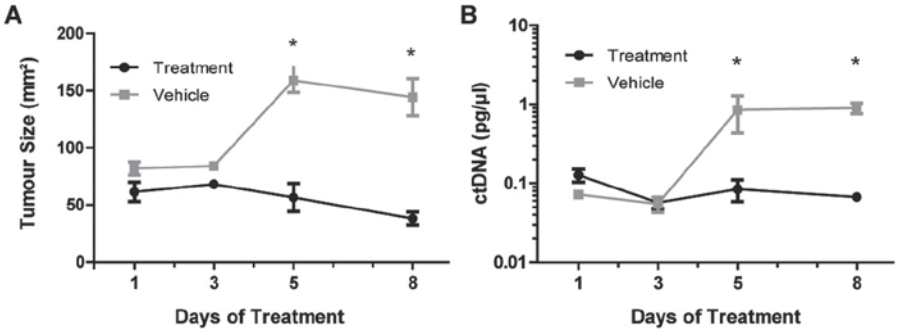

Figure 5. ctDNA kinetics during treatment. (A) 1×10^6 cells of the Colo205 line were injected subcutaneously and treatment started when tumors reached an average size of 50 mm². Animals of the vehicle group received MEK-inhibitor (BAY 869766) or vehicle substance for 1, 3, 5, or 8 days (n = 4/group). Tumor size was measured at time point of blood collection. (B) ctDNA amounts were determined in 15 µL of plasma by *AluJ* qPCR assay. ctDNA concentrations were calculated based on spiked standard rows and shown in pg/µL plasma. Data were analyzed by unpaired t-test and significant differences ($p < .05$) are indicated by asterisk.

measurements in preclinical studies. However, at least for Colo205 xenografts, the tumor size at the start of treatment should be above 100 mm² so that a decrease in ctDNA can be measured.

Cell-free DNA in clinical samples

As we could detect elevated levels of ctDNA in the preclinical setup and could show the feasibility of therapy monitoring, we wanted to test the applicability of the *AluJ* assay to assess cfDNA levels in clinical samples. Targeting human *AluJ* sequences, we were limited to the detection of total cfDNA as no tumor-specific mutations were amplified. As mutations in *KRAS* predict response towards MEK-inhibition, we analyzed samples of colorectal cancer, where the prevalence of *KRAS* mutations is 36% in the metastatic setting (Cejas et al. 2009). Plasma samples of 20 colorectal cancer patients were analyzed. We found significantly higher levels of cfDNA in cancer patients compared to healthy controls ($p < .0001$). Interestingly, the highest levels of cfDNA were found in patients positively tested for metastatic spread, showing the relevance of cfDNA in cancer patients (Figure 6).

Discussion

The potential use of circulating tumor DNA as biomarker in cancer has been tested during the last years (Fiegl et al. 2005; Diehl et al. 2008; Su et al. 2008). Preclinical models are well suited to study the release of ctDNA during disease progression and under treatment, but few

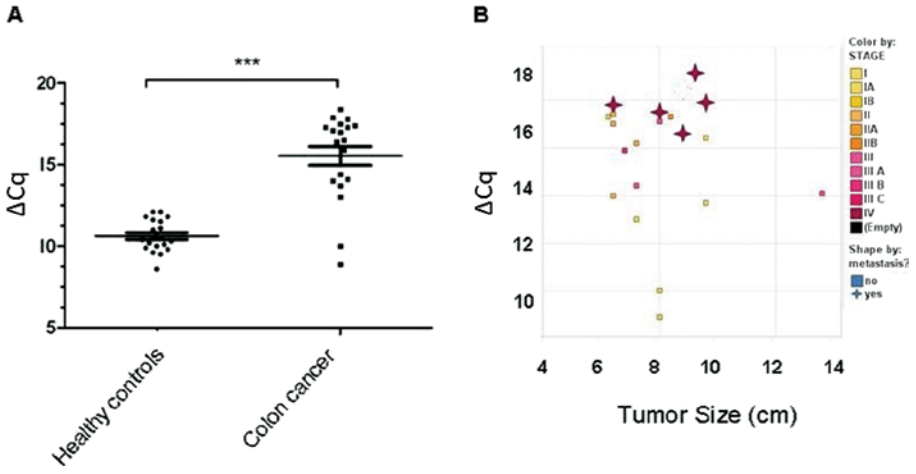

Figure 6. Analysis of cell-free DNA amount in human plasma samples. (A) Plasma DNA out of 20 healthy controls (female $n = 10$/male $n = 10$) and 20 colorectal cancer patients was purified and amplified by qPCR targeting *AluJ* sequences. ΔCq values were calculated by: Cq value of the sample subtracted from the Cq value of the negative control (H_2O) ($p < .0001$). (B) cfDNA amount was related to tumor size, tumor stage, and metastasis. Patients positively tested for metastases are marked by asterisk.

publications describe the quantification and analysis of ctDNA in xenograft models. One recent publication describes the detection of human tumor DNA in nude mice xenografted with colorectal carcinoma cells (Thierry et al. 2010). The study showed progressive increase of tumor-derived ctDNA correlating with tumor size by targeting species-specific *KRAS* and *PSAT1* DNA sequences.

Tumor-derived ctDNA has also been successfully evaluated as a biomarker for response to therapy in a xenografted ovarian carcinoma model targeting the human *β-Actin* gene (Kamat et al. 2006). However, this assay showed comparably low sensitivity.

We attempted to optimize the use of ctDNA for serial monitoring of tumor burden and response to treatment in xenograft models by targeting human-specific DNA sequences (Schneider et al. 2002; Rago et al. 2007). As the amplicon size was found to be important when quantifying ctDNA, only short sequences of the single-copy gene *GAPDH*, or the multicopy genes *hLINE* and *AluJ* were analyzed for assay validation. Although comparable amplicon lengths were tested the *AluJ* qPCR showed highest sensitivity and was chosen for further *in vivo* studies. Due to the very high copy number of these sequences (163,368 full lengths AluJ elements in the human genome (Bennett et al. 2008)), the AluJ-based assay has a detection limit of 0.2 pg/μL and is therefore sensitive enough to detect a single cell DNA content.

We measured ctDNA levels using this sensitive qPCR assay in orthotopic and subcutaneous xenograft models and showed that ctDNA concentrations are significantly higher in the plasma of xenografted mice compared to tumor-free animals. The extremely high sensitivity enabled us to quantify ctDNA in plasma volumes of only 15 μL allowing serial measurements during tumor growth. Using this approach, we demonstrated that ctDNA levels can be used to monitor the efficacy of therapy with a novel MEK-inhibitor (Iverson et al. 2009).

The release of ctDNA from tumor cells is probably due to necrotic or apoptotic cell death in the tumor. This process is known to increase in larger tumors where diffusion of nutrients and oxygen is not sufficient anymore and neoangiogenesis inadequate to sustain cell growth in all parts of the tumors (Lo et al. 1999). This might explain why it took 2 to 3 weeks of tumor growth before ctDNA signals were detectable. In addition, different cell lines displayed varying ctDNA levels at similar tumor sizes. For that reason, we conclude that the amount of released ctDNA during tumor growth is a cell line specific characteristic as the *AluJ* copy number itself is not different between the cell lines used in this study (data not shown). One can argue that each cell line has individual characteristics influencing the propensity to release a certain amount of DNA in a given period, e.g. time required between two cell divisions, the percentage of cells dying, the expression levels of DNAs in the tumor cells, and the likelihood that any living cell releases DNA. These findings might help to explain clinical results where it is known that the proportion of circulating DNA is influenced by type of cancer, tumor stage, grade, and location as reviewed by Jung et al. in 2010 (Jung et al. 2010).

Our model provides the convenience that human (tumor) DNA can easily be discriminated from host DNA by PCR targeting human *AluJ* sequences. Therefore, an application of the described *AluJ* detection assay to clinical samples faces an obstacle: human samples do not only contain ctDNA but also host-derived cfDNA, which can be detected in plasma or serum samples of healthy individuals or patients with other destructive diseases as well. A method for mutation specific discrimination and quantification of mutated tumor DNA has been published by Diehl and colleagues (Diehl et al. 2008). This BEAMing technology has been used successfully to monitor tumor dynamics by detection of specific mutations in colorectal cancer. However, this technique is dependent on specific mutations whereas testing for cfDNA is generally applicable. Several studies reported higher amounts of cfDNA in cancer patients, which is probably due

to a high rate of apoptotic and necrotic processes in cancer cells (Stroun et al. 2000; Ziegler et al. 2002; Li et al. 2003). Using the *AluJ* DNA detection method, we confirmed significantly elevated levels of cfDNA in colorectal cancer patients compared to healthy individuals. Interestingly, patients positively tested for metastatic spread showed highest levels of cfDNA confirming the results of Schwarzenbach and colleagues (Schwarzenbach et al. 2008).

Although higher cfDNA levels can be found in body fluids of cancer patients when compared to healthy individuals (Chang et al. 2002), divergent results – including similar cfDNA levels for healthy and diseased individuals – have been reported in miscellaneous studies of the same type of cancer as reviewed by Jung et al. in 2010 (Jung et al. 2010). Varying results probably arise from varying experimental procedures such as different target genes, choice of sample specimen, sample preparation, and storage. We think that the detection of *AluJ* sequences is a very useful tool to verify the value of cfDNA for therapy monitoring in the clinical situation especially in metastatic colorectal cancer where high baseline cfDNA levels would allow the detection of response to treatment with novel agents, such as the novel MEK-inhibitor BAY 869766.

Conclusion

We established a highly sensitive assay for circulating tumor DNA and found increased levels of ctDNA corresponding to the tumor size in xenograft models. In addition, the sensitivity of the assay allowed for serial monitoring in xenograft experiments. Fifteen microliters plasma samples were sufficient to allow for serial monitoring to show response to the novel MEK-inhibitor BAY 869766. In addition, using our assay we could confirm significantly elevated levels of cfDNA in colorectal cancer patients. Interestingly, highest levels were found in the metastatic situation.

The results of our study show that quantification of ctDNA is a simple and useful tool for monitoring therapy response and disease progression in animal models and might also function as response biomarker in the clinical setting. We think that xenograft mouse models are a good system to explore the effect of novel therapeutics with novel mechanism of action on ctDNA concentrations as a marker for response to therapy. In such experiments, the use of ctDNA as response biomarker has been preclinically validated and can now be used in clinical studies.

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Declaration of interest

All authors, except Johanna Schiller and Daniel Schuetzmann are full-time employees of Bayer Pharma AG.

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