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Peter B. Gahan
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Circulating Nucleic Acids in Serum and Plasma – CNAPS IX

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Preface

The current volume concerns the meeting of the 9th international conference on circulating nucleic acids in plasma and serum (CNAPS) held in Berlin, Germany, on 10–12 September 2015. The aim of the conference was to bring together clinicians, researchers, and industry working in the field that includes such aspects as methodology, basic biology, early disease identification, patient stratification, treatment monitoring, and treatment follow-up. The meeting attracted some 200 participants from 25 countries as is evidenced by the range of articles in this volume.

In one of the first published reviews on circulating nucleic acids in plasma and serum (CNAPS) that gave an almost complete overview of this field, some 440 papers published at this time were cited. Eight years later, this number has increased at least threefold so demonstrating the growing interest in the analysis and characterization of extracellular nucleic acids and their possible applications.

It was very encouraging to see so many young researchers present signifying not only the development of the CNAPS field but also its attraction to the new generation of researchers, many of whom were Ph.D. students. They are well represented in the variety of CNAPS topics in this volume. The well-established researchers were significantly present, and each working session was opened by lectures from long-time active members of the CNAPS community, Alain Thierry, Dennis Lo, Dave Hoon, and Nitzan Rosenfeld, who discussed the latest developments in their fields.

When compared to previous meetings, the section on maternal fetal CNAPS in this meeting was not as large, probably reflecting the fact that noninvasive prenatal testing (NIPT) is becoming well established in the clinical setting and has been offered commercially since the summer of 2011. In contrast, the sections on the biology of CNAPS and basic and technical applications were larger showing that although the technology is advancing, there are still a number of basic issues to be resolved, in particular, the long-standing problem of standardization to achieve a consensus on the setting of pre-analytical handling procedures for blood samples to be used for the isolation and analysis of extracellular nucleic acids. This objective was not achieved and remains an important topic for CNAPS X.

Dagmar Schmitz gave a stimulating talk on the ethical problems associated with the analysis of CNAPS. As NIPT is the first commercially available application, her talk focused primarily on the ethical issues of this field. But it is clear that her conclusions go beyond that and will have a meaning for

researchers and clinicians dealing with the analysis of nonmalignant and malignant diseases alike.

The second day of CNAPS IX concluded with a round panel discussion, "Academia Meets Industry", bringing together members from both sides. Among the topics discussed was an important aspect, namely, the strengths and weaknesses of the two "worlds" that might lead to a better understanding of each "world." A transcript of this session is included that will allow a larger audience the chance to judge this issue for itself.

Montpellier Cedex, France — Peter B. Gahan
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Part I

Nucleic Acids in Oncology – Prognosis, Treatments Screening and Metastases

1 Circulating Cell-Free miR-373, miR-200a, miR-200b and miR-200c in Patients with Epithelial Ovarian Cancer

Xiaodan Meng, Volkmar Müller, Karin Milde-Langosch, Fabian Trillsch, Klaus Pantel, and Heidi Schwarzenbach

Abstract

In the present study, we investigated whether circulating cell-free microRNAs serve as potential biomarkers in epithelial ovarian cancer (EOC) patients. Circulating miR-373, miR-200a, miR-200b and miR-200c were quantified in a cohort of 60 EOC patients, 20 patients with benign ovarian diseases and 32 healthy women using quantitative TaqMan MicroRNA assays. The serum concentrations of cell-free miR-373, miR-200a, miR-200b and miR-200c were significantly higher in EOC patients than in healthy women ($p = 0.0001$). With a sensitivity of 83 % and a specificity of 100 %, the combination of miR-200a, miR-200b and miR-200c could differ between malignant and benign ovarian tumors ($p = 0.0001$). Elevated levels of these cell-free microRNAs could be detected in FIGO I–II and FIGO III–IV stages, grading G1-2 and G3 and lymph node-negative and -positive EOC. In conclusion, the increased serum levels of this microRNA panel have diagnostic value for distinguishing healthy controls and benign tumors from EOC.

Keywords

Cell-free miRNAs • FIGO stage • Tumor progression • Metastases

Introduction

Due to its late diagnosis in advanced disease stages, epithelial ovarian cancer (EOC) is the most lethal gynecological cancer in the Western world. Approximately 70 % of patients are diagnosed with advanced FIGO stages (III or IV) and have a 5-year survival rate of less than 40 %, whereas patients who are diagnosed with FIGO stage I or II have a longer 5-year survival rate of 70–90 %. Current diagnostic methods for detec-

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tion and monitoring of EOC mainly include pelvic examination, transvaginal ultrasound and measurement of the serum biomarker CA125 (carbohydrate antigen 125) (Jayson et al. 2014). However, these methods are not sufficiently specific to diagnose EOC at an early stage since, for example, CA125 is only elevated in approximately 50 % of stage I and 70–90 % of advanced diseases (Meinhold-Heerlein and Hauptmann 2014). Therefore, based on their biological functions and their quantification in patient blood in real-time, cell-free microRNAs (miRNAs) may be a new promising class of potential non-invasive biomarkers for EOC.

Cell physiological events, such as apoptosis, necrosis and active secretion, release miRNAs into the blood circulation (Schwarzenbach et al. 2014). In the bloodstream miRNAs circulate relatively stably because either they form complexes with specific RNA-binding proteins e.g., AGO2 and HDL proteins (Arroyo et al. 2011) or are integrated in apoptotic bodies or microvesicles (Rani 2014). MiRNAs are a family of evolutionary conserved, small non-coding RNA molecules consisting of approximately 22 nucleotides. They inhibit gene expression post-transcriptionally by binding specifically to the 3' untranslated-region (3'UTR) of their target mRNA (Bartel 2009). They are believed to regulate approximately 50 % of all protein-coding genes. Computational analyses indicate that one miRNA has binding affinity to hundreds of different mRNAs and hence, miRNAs are involved in the regulation of various cellular processes, such as development, differentiation and proliferation. (Krol et al. 2010). As miRNA loci frequently map to fragile chromosomal regions harboring DNA amplifications, deletions or translocations, their expression is often deregulated during tumorigenesis, so contributing to tumor progression and metastasis (Calin et al. 2004).

Materials and Methods

Populations Studied

Serum samples from 60 EOC patients treated at the University Medical Center Hamburg-Eppendorf, Department of Gynecology, for histologically confirmed International Federation of Gynecology and Obstetrics (FIGO) stages I-IV, 20 patients with benign ovarian diseases and 32 healthy women with no history of cancer and in good health based on self-report were included in the present study. Median ages of EOC patients, patients with benign ovarian diseases and healthy women were 56, 46 and 56, and ranged from 23 to 79, from 17 to 74 and from 42 to 70, respectively. Serum samples of EOC patients were collected directly before surgery from June 2013 to May 2015. Those of patients with benign ovarian tumors and healthy women were obtained during 2014–2015. All patients gave written informed consent to access their blood and review their medical records according to our investigational review board and ethics committee guidelines. Regarding blood processing, uniform management concerning the specific, described protocols was performed. Detailed patient characteristics are summarized in Table 1.1.

Table 1.1 Patient characteristics at the time of primary diagnosis of EOC or benign diseases

	EOC	Benign	Healthy
	(n = 60)	**(n = 20)**	**(n = 32)**
Age			
Median (range)	56 (23–79)	46 (17–74)	56 (42–70)
FIGO stage			
FIGO I–II	8 (13.3 %)		
FIGO III	34 (56.7 %)		
FIGO IV	7 (11.7 %)		
unknown	11 (18.3 %)		
Grading			
G1, G2	4 (6.6 %)		
G3	43 (71.7 %)		
unknown	13 (21.7 %)		
Lymph node metastasis			
N0	13 (21.7 %)		
N1	27 (45.0 %)		
unknown	20 (33.3 %)		
Histologic subtype			
Serous papillary	43 (71.7 %)		
Other subtypes	2 (3.3 %)		
unknown	15 (25.0 %)		

Extraction of Cell-Free RNA and Conversion into cDNA

Cell-free RNA was extracted with the mirVana PARIS kit (Life Technologies, New York, USA) from 600 µl serum according to the manufacturer's instructions. The RNA was quantified on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and immediately reverse transcribed into cDNA by the TaqMan miRNA Reverse Transcription Kit (Life Technologies, New York, USA). For extraction efficiency, 20 fmol of synthetic non-human cel-miR-39 was added as an exogenous spike control.

Quantitative Real-Time PCR of miR-373, miR-200a, miR-200b, miR-200c

Quantitative real-time PCR was performed using miRNA-specific TaqMan miRNA assays (Life Technologies) for miR-484 (reference miRNA), miR-373, miR-200a, miR-200b, miR-200c and according to the manufacturer's instructions. The quantitative real-time PCR reaction was performed at 95 °C for 10 min. and in 40 cycles at 95 °C for 15 s and 60 °C for 60 s on a C1000 Touch real-time PCR device (Bio-Rad, California, USA).

MiR-484 was chosen as a reference gene to normalize the miRNA data, because this miRNA showed the smallest variation between healthy individuals, patients with benign ovarian diseases and EOC patients. In the serum samples of healthy women, patients with benign ovarian diseases and EOC patients, the mean values of cell-free miR-484 were 15.12 (SD = 0.86), 13.07 (SD = 1.36) and 15.91 (SD = 1.88), respectively. The inter-individual variability of the efficiency of our procedures was also controlled by spiking with cel-miR-39. Our measurements showed a mean value of 15.27 (SD = 1.13) and a median value of 15.05, indicating that our data are relatively robust.

The obtained data for the miRNA expression levels were calculated and evaluated by the ΔCt method as follows: ΔCt = mean value Ct (reference miR-484) – mean value Ct (miRNA of interest), and the relative miR levels corresponded to the value of 2^(ΔCt).

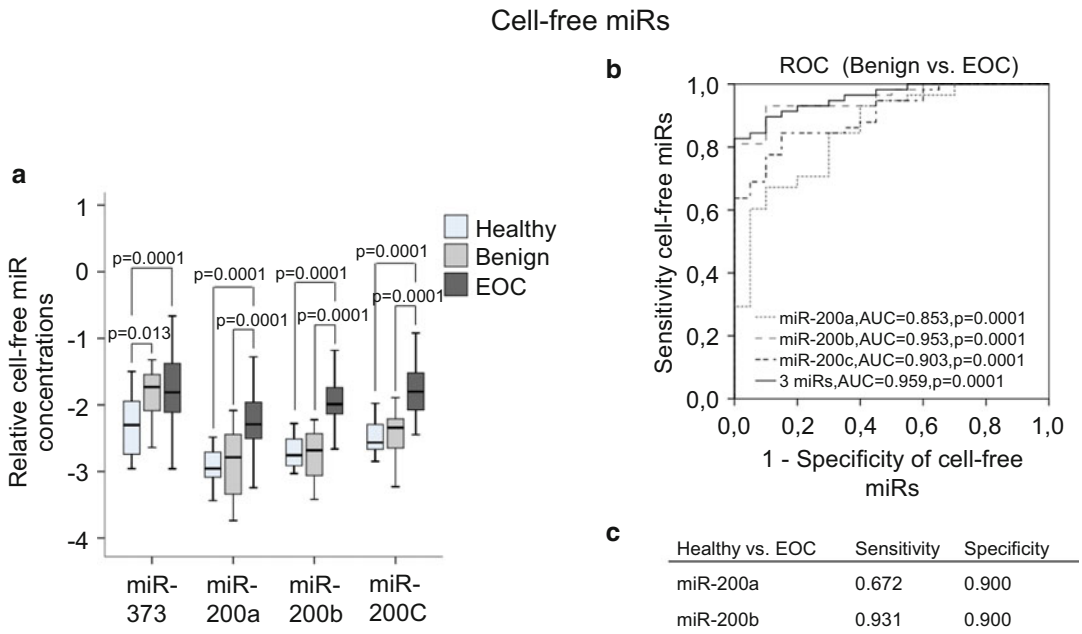
To avoid false positive data (e.g., primer dimer formation or nonspecific PCR products), a negative control without any templates was included from the starting point of reverse transcription. To check whether the PCR products are real amplification products, we analyzed them by agarose gel electrophoresis.

Statistical Analysis

The statistical analyses were performed using the SPSS software package, version 22.0 (SPSS Inc. Chicago, IL). Relative expression data were log10 transformed in order to obtain normal distribution data. Statistical differences of miRNA expressions between healthy controls, patients with benign ovarian diseases and EOC patients were calculated using ANOVA with Tukey's HSD test for all pairwise comparisons that correct for experiment-wise error rate. The diagnostic power of the miRNAs was analyzed by receiver operating characteristic (ROC) curves. Areas under the curves (AUC) were calculated, assuming nonparametric distribution. Binary logistic regression was performed to obtain the probabilities of combined miRNAs and to perform the ROC analysis. Missing data were handled by pairwise deletion. A p-value <0.05 was considered as statistically significant. All p-values are two-sided.

Results

We quantified cell-free miR-373, miR-200a, miR-200b and miR-200c in the serum of 60 EOC patients, 20 patients with benign ovarian diseases and 32 healthy women. As shown in Fig. 1.1a, the levels of cell-free miR-373, miR-200a, miR-200b and miR-200c in the serum of EOC patients were higher than those in healthy women ($p = 0.0001$). We observed a significant difference in the serum levels of miR-200a, miR-200b and miR-200c between EOC patients and patients



c

Healthy vs. EOC	Sensitivity	Specificity
miR-200a	0.672	0.900
miR-200b	0.931	0.900
miR-200c	0.845	0.850
3 miRs	0.828	1.000

Fig. 1.1 Comparison of serum levels of miR-373, miR-200a, miR-200b, miR-200c in healthy women (n = 32), patients with benign ovarian diseases (n = 20) and EOC patients (n = 60) (**a**). ROC analyses of the profiles of sensitivity and specificity of cell-free miR-200a, miR-200b, miR-200c and their combination to distinguish benign ovarian tumors from EOC (**b**). Table of sensitivities and specificities (**c**)

with benign ovarian diseases (Fig. 1.1a, p = 0.0001), with AUC values of 0.853, 0.953 and 0.903 respectively, showing their cancer-specific increase (Fig. 1.1b). To improve the discrimination between patients with malignant and benign tumors, the concentrations of these miRNAs were combined. This resulted in a sensitivity of 83 % and a specificity of 100 % (Fig. 1.1c).

In addition, we compared the concentrations of these cell-free miRNAs in the serum of EOC patients with their clinical and histopathological risk factors. The serum levels of cell-free miR-373 were only upregulated in FIGO III-IV, whereas those of miR-200a (p = 0.0001, p = 0.0001), miR-200b (p = 0.0001, p = 0.0001) and miR-200c (p = 0.007, p = 0.0001) were increased in FIGO I–II and III–IV stages, respectively (Fig. 1.2a). We detected similar results in lymph node-negative and -positive patients as well as in the low (G1–2) and the high (G3) grade patient subgroups (Fig. 1.2c).

Discussion

In the present study, we detected increased serum levels of cell-free miR-373, miR-200a, miR-200b and miR-200c in all tumor stages of EOC patients. The levels of miR-200a, miR-200b and miR-200c could differ between malignant and benign ovarian tumors with a sensitivity of 83 % and a specificity of 100 %.

MiR-200a, miR-200b and miR-200c belonging to the miR-200 family have been reported to be down- or up-regulated in cancer. This discrepancy has been explained by their multiple characteristics (Muralidhar and Barbolina 2015). They play a major role in the suppression of epithelial-

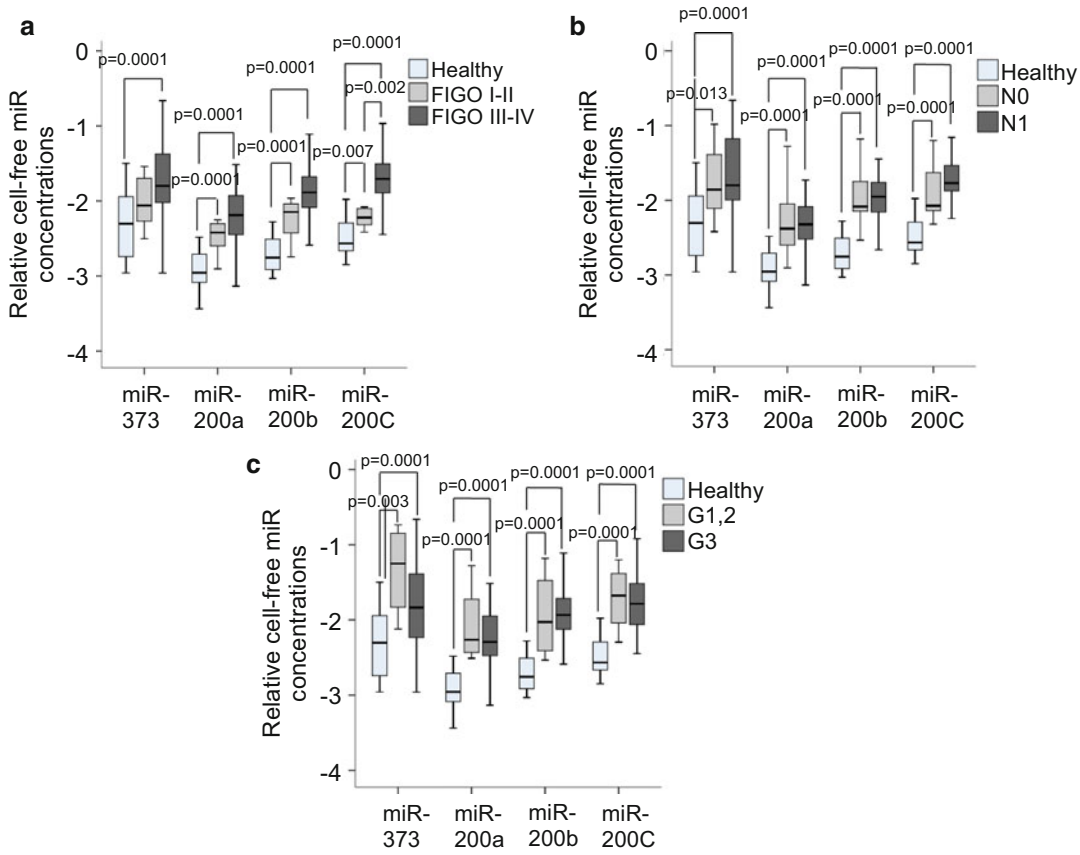


Fig. 1.2 Comparison of serum levels of miR-373, miR-200a, miR-200b, miR-200c in healthy women (n = 32), EOC patients with FIGO I–II (n = 8) and FIGO III–IV (n = 41) (**a**), lymph-node negative (N0, n = 13) and lymph-node positive (N1, n = 27) EOC patients (**b**) and low (G1, G2, n = 4) and high grade (G3, n = 43) EOC patients (**c**)

to-mesenchymal transition (EMT) and metastasis (Muralidhar and Barbolina 2015; Koutsaki et al. 2014). During EMT epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties by down-regulating E-cadherin and upregulating vimentin expression (Park et al. 2008). MiR-200 family members were found to directly target the mRNA of the E-cadherin transcriptional repressors ZEB1 and ZEB2 that are crucial inducers of EMT in various human tumors (Hu et al. 2009). In this regard, low-level expression of miR-200a and miR-200b in advanced EOC significantly correlated with cancer recurrence and poor overall survival while over-expression of the miR-200 cluster inhibited EOC cell migration (Hu et al. 2009). In contrast, it has also been reported that an elevated expression of these miRNAs is a significant characteristic of EOC because their genes show frequently copy number gains in cancer (Zhang et al. 2006). Their up-regulation was associated with aggressive tumor progression and could predict prognosis and survival in EOC patients (Kan et al. 2012). Our data complement these findings and show a cancer-specific up-regulation of these miRNAs in all tumor stages of EOC patients. They could distinguish between malignant and benign ovarian tumors with a high sensitivity and specificity.

Previous findings provoked us to additionally investigate the serum levels of cell-free miR-373 in EOC patients that is also involved in EMT (Chen et al. 2015). Recently, we showed that increased concentrations of miR-373 are associated with negative receptor status of breast cancer patients, and its over-expression both down-regulated the protein expression of estrogen

receptors and inhibited apoptosis in the breast adenocarcinoma cell line MCF-7 (Eichelser et al. 2013, 2014). Moreover, we observed a specific influence of neo-adjuvant therapy on the serum levels of miR-373 in these patients (Muller et al. 2014). Here, we show the up-regulated expression of miR-373 in both malignant and benign tumors. Its levels were elevated in all FIGO stages and independent of lymph node status. Similar to the miR-200 family, miR-373 has also been described as an oncogene and a tumor suppressor gene. MiR-373 either promoted proliferation in EOC cells (Nakano et al. 2013) or inhibited EOC invasion and metastasis (Zhang et al. 2014).

In conclusion, our data show the diagnostic relevance of serum miR-200a, miR-200b, miR-200c and miR-373 in EOC patients.

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Cell-Free miRNA-141 and miRNA-205 as Prostate Cancer Biomarkers

2

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Abstract

Expression levels of five miRNAs (miR-19b, miR-21, miR-126, miR-141, miR-205) were measured in the plasma of healthy donors and prostate cancer patients. It was shown that miR-141 expression level efficiently discriminates early stage prostate cancer patients and correlates with the Gleason score.

Keywords

miRNA • Prostate cancer • Biomarkers

Introduction

Prostate cancer (PCa) is one of the widespread male malignancies worldwide. Despite high 5-year survival rates, efficient, non-invasive, differential PCa diagnostics, including tumor staging and post therapy monitoring are demanded. Actually, PSA based PCa diagnostics has low specificity and is not recommended by USPSTF whereas a number of circulating blood biomarkers, including miRNA, were shown to be useful for non-invasive cancer

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diagnostics (Ralla et al. 2004). The aim of this study was to evaluate the diagnostic efficacy of cell-free miRNAs for staging and prediction of PCa outcome.

Materials and Methods

Blood samples from 47 healthy donors (HD) were obtained from the Center of New Medical Technologies (Novosibirsk, Russia) and the Novosibirsk Research Institute of Circulation Pathology (Novosibirsk, Russia). Samples from 48 prostate cancer (PC) patients were obtained from the Novosibirsk Regional Oncologic Dispensary (Novosibirsk, Russia). All blood samples were collected between January 2014 and March 2015. The study was performed in accordance with current regulations, approved by the ethics committees of all participating institutions and written informed consent was provided by all participants.

Circulating miRNAs were isolated from blood plasma by precipitation of proteins by the acetate buffer method (Lechnov et al. 2015) followed by purification on silica-based columns. Expression levels of miR-19b, miR-21, miR-126, miR-141, miR-205 were measured by qRT-PCR (Chen et al. 2005) and normalized to the mean value of miR-16 and miR-101.

Results and Discussion

Two of the five miRNAs (miR-141, miR-205) were significantly upregulated in PCa patients ($P < 0{,}0001$, *T*-test, two-tailed) versus HD.

ROC curve analysis of these miRNA expressions in PCa versus HD demonstrate that miR-141 discriminates groups with 100 % specificity and 56,25 % sensitivity (Fig. 2.1, Panel A). MiRNA-205 was shown to be less efficient demonstrating 66,7 % and 77,3 % sensitivity and specificity. Binary logistic regression of both miRNAs, demonstrates that the combination of the two miRNAs showed the highest predictive value (Fig. 2.1, Panel B).

Expression levels miR-141 and miR-205 depend on the clinico-pathologic characteristics studied. PCa patients were assigned to groups according to either the Gleason score (19 patients with Gleason score 6 and 21 patients with Gleason score 7) or the TNM-classification (9 patients with T2a-2b stage, 14 with T2c, 10 with T3a and 9 with T3b stage).

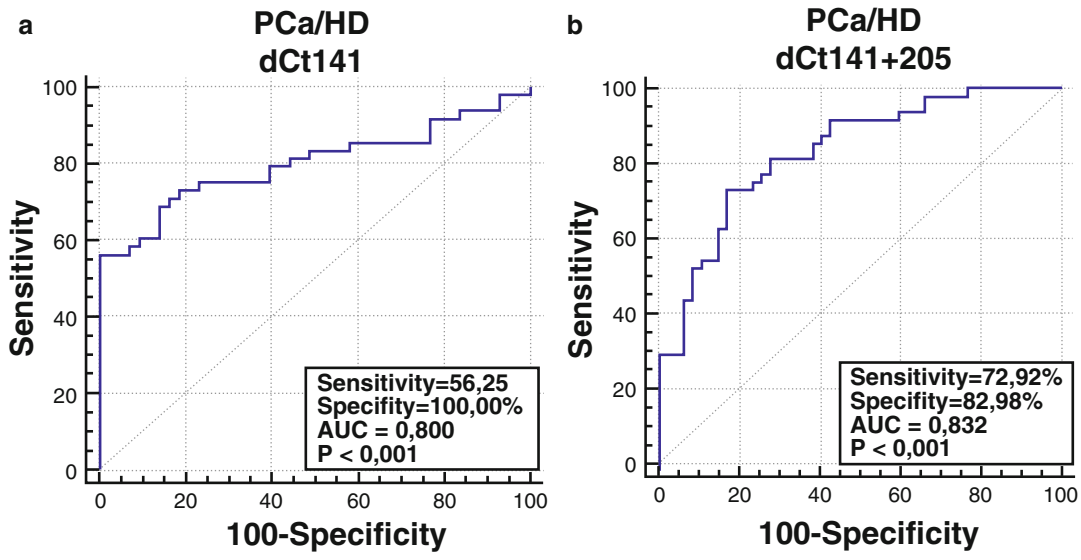

Fig. 2.1 ROC curves for miR-141 and binary logistic regression of both miRNAs

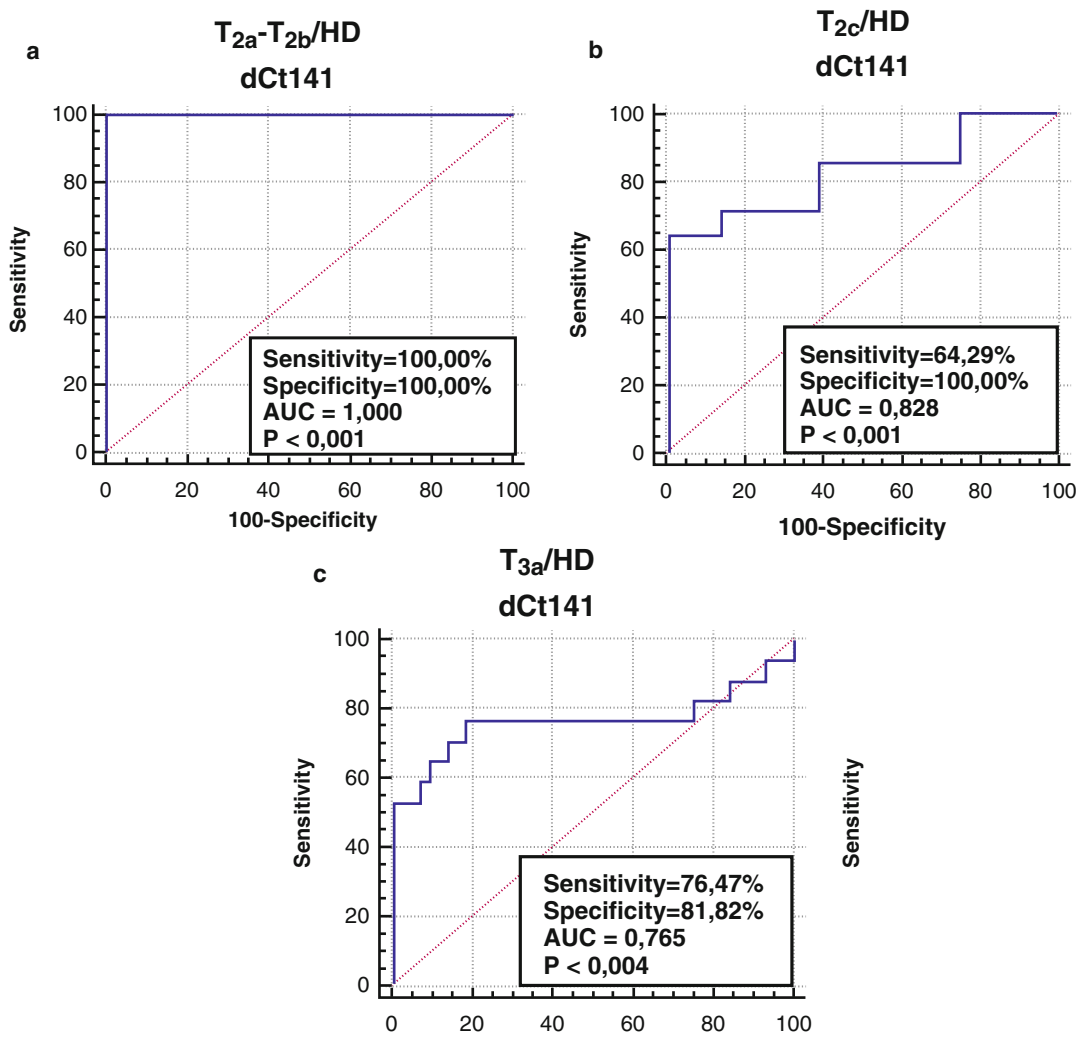

Fig. 2.2 ROC –analysis of miR-141 discrimination efficacy

MiR-141 showed meaningful differences between groups of patients with Gleason scores of 6 and 7 (P < 0,05, two-tailed *T*-test), Gleason score 6 and HD (P < 0,0001, two-tailed *T*-test) and Gleason score 7 and HD (P < 0,05, two-tailed *T*-test). ROC-analysis demonstrates that miR-141 efficiently discriminates HD from Gleason score 6 PCa patients (sensitivity 93,62 %, specificity 73,68 %, AUC = 0868) and less efficiently, HD from Gleason score 7 patients (sensitivity = 72,34 %, specificity = 71,43 %, AUC = 0718).

Analysis of miR-141 expression in the plasma of T2a-2b stage PCa patients enables discrimination between these patients and HD with 100 % specificity and sensitivity while PCa patients at the later stages could not be diagnosed with similar efficacy (Fig. 2.2).

MiRNA-205 expression showed no dependence on the stage of PCa. Totally, the data obtained demonstrate efficient discrimination of the PCa patients at the early stages of the disease and correlation of miR-141 expression with the

Gleason score. To confirm the diagnostic efficacy of miR-141 patients with benign prostatic hyperplasia and non-cancer related inflammation is planned to be included in a testi cohort list.

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Conflict of Interest The authors declare no conflict of interest.

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3 Clinical Utility of Circulating Tumor DNA for Molecular Assessment and Precision Medicine in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal malignancies. The genomic landscape of the PDAC genome features four frequently mutated genes (*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*) and dozens of candidate driver genes altered at low frequency, including potential clinical targets. Circulating cell-free DNA (cfDNA) is a promising resource to detect molecular characteristics of tumors, supporting the concept of "liquid biopsy".

We determined the mutational status of *KRAS* in plasma cfDNA using multiplex droplet digital PCR in 259 patients with PDAC, retrospectively. Furthermore, we constructed a novel modified SureSelect-KAPA-Illumina platform and an original panel of 60 genes. We then performed targeted deep sequencing of cfDNA in 48 patients who had ≥1 % mutant allele frequencies of *KRAS* in plasma cfDNA.

Droplet digital PCR detected *KRAS* mutations in plasma cfDNA in 63 of 107 (58.9 %) patients with inoperable tumors. Importantly, potentially targetable somatic mutations were identified in 14 of 48 patients (29.2 %) examined by cfDNA sequencing.

Our two-step approach with plasma cfDNA, combining droplet digital PCR and targeted deep sequencing, is a feasible clinical approach. Assessment of mutations in plasma cfDNA may provide a new diagnostic tool, assisting decisions for optimal therapeutic strategies for PDAC patients.

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Keywords

Plasma cfDNA • Targeted deep sequencing • Pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease and the seventh leading cause of cancer death in the world. Despite a rising incidence worldwide, tools for early detection remain unavailable and the prognosis is poor with a 5-year survival rate limited to approximately 4–7 %. To date, surgery is the only curative treatment for PDAC, but only 15–20 % of patients present with localized, non-metastatic disease. The rapid organ metastasis observed in some patients treated with potentially curative surgery suggests that occult distant metastases may happen very early, negating survival benefit in the subset of operable patients.

Whole-genome or exome sequencing analyses have revealed that somatic alterations of the PDAC genome often occur in four genes, namely *KRAS*, *CDKN2A*, *TP53* and *SMAD4* (Waddell et al. 2015; Biankin et al. 2012; Jones et al. 2008) and at low frequency in dozens of candidate driver genes. Jones and colleagues have determined driver genes in PDAC that are associated with U.S. Food and Drug Administration (FDA)-approved therapies for oncologic indications or therapies in published prospective clinical studies (Jones et al. 2015). They identified somatic alterations in genes with target potential in approximately 20 % of PDAC patients. This implies the possibility of precision medicine strategies using genomic profiling in PDAC.

Tumor-derived DNA in cell-free DNA (cfDNA), also known as circulating tumor DNA (ctDNA) provides a less-invasive approach to diagnose cancers based on the concept of "liquid biopsy". While detection of ctDNA is still challenging since only a small fraction of total cfDNA is derived from tumor cells, advances in technology are making it more feasible. Especially, picoliter-droplet digital PCR (ddPCR) technologies (RainDance Technologies) possess high sensitivity and allow multiplex assays. A NGS approach is more suitable to detect a wide range of cancer-related gene mutations than ddPCR. Because mutations in *KRAS* are among the first to occur during carcinogenesis and they are observed in >90 % of PDAC, we first evaluated *KRAS* mutations in plasma as a benchmark of ctDNA from 259 patients with PDAC and then analyzed correlations with clinicopathological features. In addition, we performed targeted deep sequencing of cfDNA using the SureSelect-KAPA-Illumina platform to detect potentially therapeutically targetable mutations and copy number alterations in PDAC patients.

Materials and Methods

Plasma cfDNA was extracted from 2 mL of plasma samples using the QIAsymphony system or QIAamp Circulating Nucleic Acid Kit (QIAGEN). Before DNA extraction, plasma samples were centrifuged at 16,000 g for 10 min at 4 °C in order to remove cell debris. Eluted cfDNA was quantified by quantitative PCR of human LINE-1 sequences. Multiplex ddPCR assays were performed using cfDNA corresponding to 250 μL of plasma and the RainDrop digital PCR system (RainDance Technologies). For targeted sequencing analysis, sequence libraries were prepared using a combination of a KAPA Hyper Prep Kit (Kapa Biosystems) and the SureSelect Target Enrichment System (Agilent Technologies). Sequencing was performed on an Illumina HiSeq 2000 (Illumina). Somatic copy number alteration in the primary tumor was analyzed by SurePrint G3 Human CGH Microarray 1x1M and the Genomics Workbench software (Agilent technologies).

Results and Discussion

Detection of KRAS Mutations in cfDNA by ddPCR

In this study, plasma samples from 259 PDAC patients were analyzed. Clinicopathological features of patients are summarized in Table 3.1. Peripheral, venous blood samples were obtained before patients received any treatment. DNA was extracted from 2 mL plasma samples and the amount of amplifiable cfDNA was quantified by a real-time PCR-based method. It is known that >90% of PDAC harbor mutations in the *KRAS* gene as founder mutations. Therefore, the presence of mutant *KRAS* DNA fragments in plasma from PDAC patients indicates the presence of ctDNA. A multiplex ddPCR assay was created to detect the four common *KRAS* mutations (G12D, G12V, G12R and G13D), which account for 90% of all mutations of *KRAS* in PDAC. Among 151 inoperable patients, mutant *KRAS* was detected in 63 of 107 (58.9%) PDAC patients with distant organ metastasis, while only 8 of 44 (18.2%) patients without distant organ metastasis showed detectable levels of mutant *KRAS*. The presence of ctDNA was significantly ($P<0.0001$) associated with the presence of distant organ metastasis.

Table 3.1 Clinicopathological data of the patients

	n	%
Gender		
Male	160	61.8
Female	99	38.2
Age (years)		
Mean	64.7	
Median	66	
Range	24–86	
Tumor size (mm)		
Mean	40.4	
Median	37	
Range	5–115	
Tumor location		
Head	119	45.9
Body/tail	140	54.1
T factor (UICC)		
T1	5	1.9
T2	6	2.3
T3	160	61.8
T4	81	31.3
Tx	7	2.7
N factor (UICC)		
N0	142	54.8
N1	115	44.4
Nx	2	0.8
M factor (UICC)		
M0	49	18.9
M1	208	80.3
Mx	2	0.8
UICC stage		
IA	3	1.2
IB	2	0.8
IIA	29	11.2
IIB	44	17.0
III	17	6.6
IV	163	62.9
Unknown	1	0.4

We also analyzed the implications of ctDNA for clinical outcome and found that the presence of mutant *KRAS* in plasma was significantly associated with poor overall survival ($P<0.0001$). In resectable PDAC patients, 9 of 108 (8.3%) had detectable levels of *KRAS* mutation in plasma cfDNA. Among them, five patients relapsed with metastatic tumors within 6 months after surgery and had a very poor prognosis. In a representative case, the patient was diagnosed with a 19 mm localized tumor and no distant organ metastasis based on radiographic findings at the operation. However, a metastatic tumor in the liver was found by first follow-up CT 3 months after resection of the primary carcinoma and the patient died after 9 months. We evaluated the plasma sample obtained before surgery and detected 4.3% *KRAS* G12V. These findings indicated that detecting ctDNA might be useful to monitor tiny distant metastases that are, otherwise, hard to detect by routine medical imaging.

Targeted Deep Sequencing of cfDNA with the Illumina Platform

We next performed targeted deep sequencing to further analyze mutated genes in cfDNA from PDAC patients using the Illumina sequencing platform. To apply the SureSelect target enrichment system for Illumina paired-end sequencing of cfDNA, we optimized the library preparation con-

ditions so that we could successfully reduce DNA sample input from 200 ng (the minimum input according to the manufacturer's protocol) to "10 ng" by modifying SureSelect libraries for target enrichment in combination with the KAPA Hyper Prep Kit. The sensitivity of detection of somatically mutated genes in plasma appeared to be dependent on the amount of cfDNA, the allele frequency of the mutations in the samples and the sequencing coverage. Therefore, we focused on samples with a relatively high (≥1%) *KRAS* mutant allele frequency in plasma cfDNA. Based on the results of *KRAS* multiplex ddPCR, 43 samples probably having substantial levels of ctDNA were selected for targeted deep sequencing. In addition, five cases were included even though they were negative for *KRAS* mutation by ddPCR, since they had distant organ metastases. In order to achieve

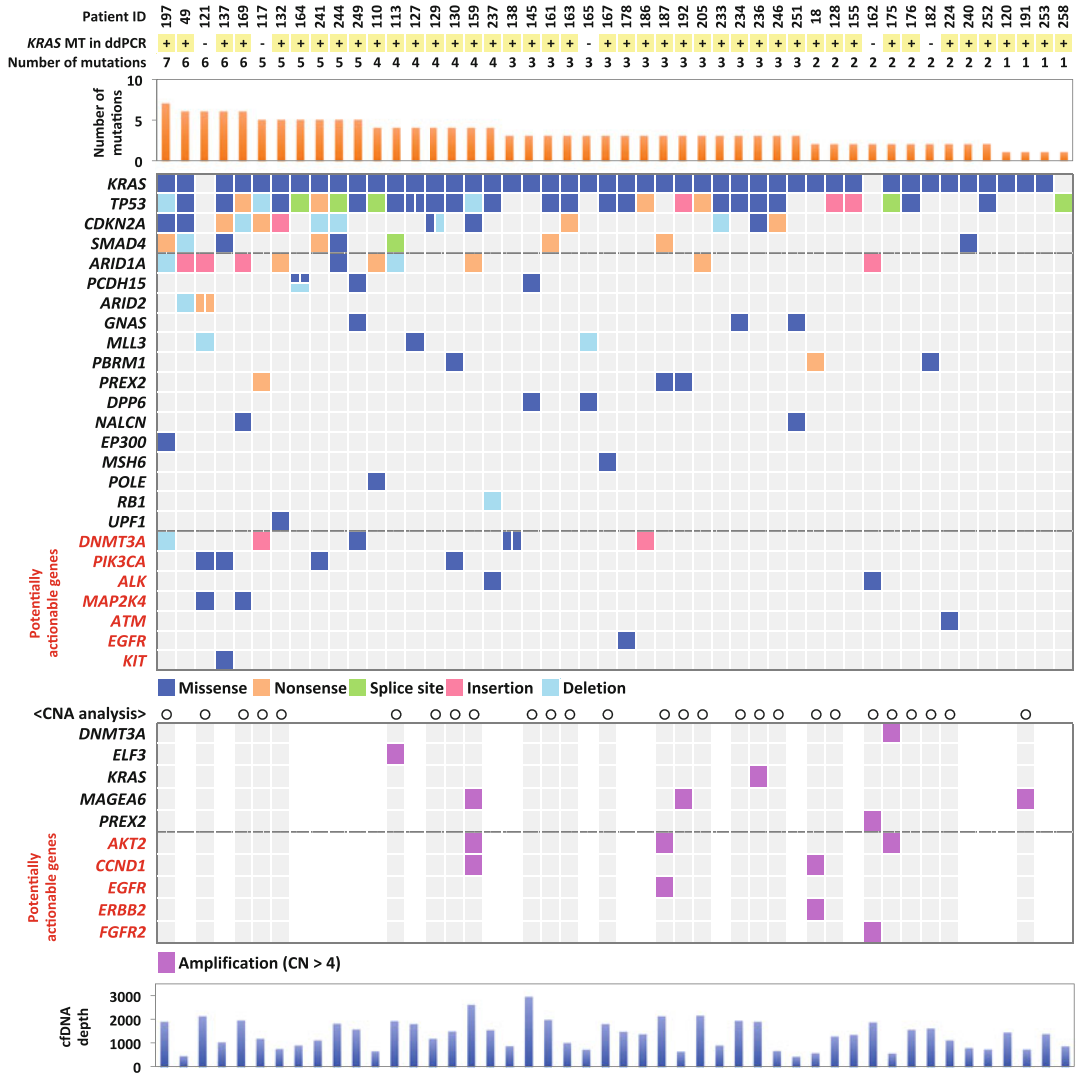

Fig. 3.1 Somatic gene alterations detected by targeted sequencing of cfDNA. The *top bar plot* shows the number of somatic mutations among 60 genes for ctDNA from each patient. The altered genes detected by targeted deep sequencing analysis are shown in the *left*-most column. The somatic mutations in 48 patients and the somatic copy number gains (copy number >4) in 27 patients with >10% tumor variant allele frequencies are shown. The *bottom bar plot* shows the averages of the coverage depth for each cfDNA sample. *CN* copy number, *CNA* copy number alteration, *MT* mutation, *NA* not applicable

higher sequence coverage (~1000x on average) cost-effectively and to test clinical feasibility, we designed an original gene panel for PDAC, focusing on 60 genes, including potential therapeutic targets. All 96 DNA libraries generated from 48 cfDNA and 48 matched germ-line DNA samples were pooled into one Flow Cell Illumina HiSeq2000. The unique coverage depth for cfDNA samples was 1356x on average (range 417–2955x), and that for the germ-line DNA samples was 1492x on average (range 948–2299x) after excluding PCR duplication. Mutations detected by targeted deep sequencing are presented in Fig. 3.1. All 48 patients had at least one mutation (median 3 mutations/patient, range 1–7 mutations).

Importantly, mutations in potential therapeutic target genes were detected in 14 of 48 (29.2 %) patients. These included *ALK* (n = 2), *ATM* (n = 1), *DNMT3A* (n = 5), *EGFR* (n = 1), *KIT* (n = 1), *MAP2K4* (n = 2), and *PIK3CA* (n = 4). In particular, three of four *PIK3CA* mutations (p.H1047L, p.E545K and p.Q546K) and one *EGFR* mutation (p.G724S) are known to be oncogenic and are *in vitro* therapeutic targets (Cho et al. 2014; Kang et al. 2005; Meyer et al. 2013).

We also analyzed somatic copy number alterations based on the targeted sequencing data using the in-house algorithm. In our algorithm, the copy number of each gene was adjusted for tumor purity of ctDNA in plasma cfDNA. Patients with low tumor variant allele frequencies (≤10 %) in plasma cfDNA were excluded. The estimated somatic copy number gains (amplifications) in 27 patients are shown in Fig. 3.1. In one case (Patient ID-18), the results were compared with copy number alterations derived from array comparative genomic hybridization (aCGH) data obtained with frozen samples of the primary cancer and matched normal tissue. Among 60 genes selected for targeted sequencing, amplifications of the *CCND1* and *ERBB2* genes were detected in both ctDNA and the primary cancer in this patient. Amplifications of *CCND1* and *ERBB2* genes are potentially targetable (Finn et al. 2015; Aumayr et al. 2014).

In conclusion, we propose a novel approach for PDAC liquid biopsies: first analyze *KRAS* alleles using ddPCR as a benchmark and confirm the presence of sufficient ctDNA, and secondly, perform targeted sequencing of genes mutated frequently in PDAC and potential therapeutic target genes in patients with mutant *KRAS* alleles detected by ddPCR or patients with advanced PDAC with distant organ metastasis. We believe our approach could be cost-effective and applicable in the clinic. Finally, assessment of mutations and copy number alterations of plasma cfDNA may provide a new prognostic tool that would be helpful in deciding optimal therapeutic strategies for PDAC patients. Evaluation of ctDNA could be incorporated into future clinical trials as an alternative to primary or metastatic tumor biopsies for precision medicine in cancer cases.

Conflict of Interest The authors have declared no conflicts of interest.

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4 An Enquiry Concerning the Characteristics of Cell-Free DNA Released by Cultured Cancer Cells

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Abstract

Non-invasive screening that utilizes cell-free DNA (cfDNA) offers remarkable potential as a method for the early detection of genetic disorders and a wide variety of cancers. Unfortunately, one of the most prominent elements delaying the translation of cfDNA analyses to clinical practice is the lack of knowledge regarding its origin and composition. The elucidation of the origin of cfDNA is complicated by the apparently arbitrary variability of quantitative and qualitative characteristics of cfDNA in the blood of healthy as well as diseased individuals. These factors may contribute to false positive/negative results when applied to clinical pathology. Although many have acknowledged that this is a major problem, few have addressed it. We believe that many of the current difficulties encountered in *in vivo* cfDNA studies can be partially circumvented by *in vitro* models. The results obtained in this study indicate that the release of cfDNA from 143B cells is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily the result of actively released DNA, perhaps in association with a protein complex. Moreover, this study demonstrates the potential of *in vitro* cell culture models to obtain useful information about the phenomenon of cfDNA.

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Keywords

Cell-free DNA (cfDNA) • Apoptosis • Necrosis • Osteosarcoma • Flow cytometry

Introduction

Understanding the processes involved in the generation of cell-free DNA (cfDNA) is critical for deducing its role in biology and pathology, while advancing the translation of analyses to clinical practice. However, the origin of cfDNA still remains elusive despite the seemingly universality of it in bio-fluids. Several sources of cfDNA have been excluded including, exogenous DNA (bacterial, viral and parasitic), lysis of cells on the interface between a tumour and circulation (Sorenson 1997) and the destruction of tumour micrometastases and circulating cancer cells (Bevilacqua et al. 1998). Currently, the only remaining conceivable sources that may account for the occurrence of cfDNA are apoptosis, necrosis, or active cellular secretion (Stroun et al. 2001).

Although most evidence suggests that the release of cfDNA is mainly a consequence of apoptosis, many studies have indicated that a significant fraction of cfDNA is derived from active cellular secretions (van der Vaart and Pretorius 2008), where newly synthesized DNA in association with a lipid-protein complex is released in a homeostatic manner (Anker et al. 1975; Borenstein and Ephrati-Elizur 1969; Stroun and Anker 1972; Stroun et al. 1977, 1978). The aim of this work was to examine the release and composition of cfDNA from cultured human osteosarcoma cells (143B). The release pattern of cfDNA was characterized over time, and the sizes of the cfDNA fragments evaluated at each of these intervals. Additionally, apoptotic, necrotic and the cell cycle profiles were also investigated using flow cytometry.

Materials and Methods

Cell Culturing, Extraction and Quantification of Cellular Protein and Cell-Free DNA

The human osteosarcoma cell line 143B was obtained from the American Type Culture Collection (ATCC® CRL- 8303TM). Cells were cultured as prescribed by ATCC. Total cellular protein was isolated and quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies). cfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the manufacturer's PCR clean-up instructions and quantified using real-time quantitative PCR assay for the β-globin gene.

Fragment Size Evaluation of Cell-Free DNA

The size of cfDNA extracted at the different time intervals were analysed by capillary electrophoresis using the High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) equipped with Expert 2100 software. The assay was performed according to the instructions provided by the manufacturer.

Flow Cytometric Assays

The BD Annexin V FITC assay (BD Biosciences) was utilized to determine the apoptotic/necrotic profile of 143B cells. An

APO-BrdU™ Tunel assay kit (Molecular Probes, Invitrogen) was used for the detection of DNA fragments as recommended by the manufacturer. For cell cycle analysis the Click-iT® Edu Alexa Fluor® 488 Cell Proliferation Kit (Molecular Probes, Invitrogen) was utilized according to the instructions of the manufacturer. All flow cytometric analyses were done on a FACSVerseTM bench top flow cytometer. Events were acquired on BD FACSuite™ software (Becton & Dickson, Mountain view, CA, USA).

Results and Discussion

In this study, the release of cfDNA from cultured cells was evaluated in order to gauge its potential use for elucidating the nature of cfDNA. Release of cfDNA from 143B cultured cells was characterized over time (Fig. 4.1a, b).

After growth medium renewal, the amount of cfDNA increased incrementally, notably peaked after 24 h, and plateaued at a much lower level thereafter. Since the amount of cells increase over time, this is not surprising. However, when the values were normalized in terms of total cellular protein the tendency did not change, suggesting that more cfDNA is released per cell in a time dependent manner (Fig. 4.1d, c). This suggested that the cfDNA is neither from apoptotic nor necrotic origin but originates from active release. To examine the size of cfDNA isolated at the different time points, samples were subject to capillary electrophoresis (Fig. 4.2). After 4 h of incubation a prominent peak at 166 bp, however, this peak dissipated incrementally and disappeared after 24 h when a new peak of approximately 2000 bp dominated the scene. After 40 h of incubation, the cfDNA resembles multiples of nucleosomal repeats, with small populations forming at approximately 160, 340 and 540 bp,

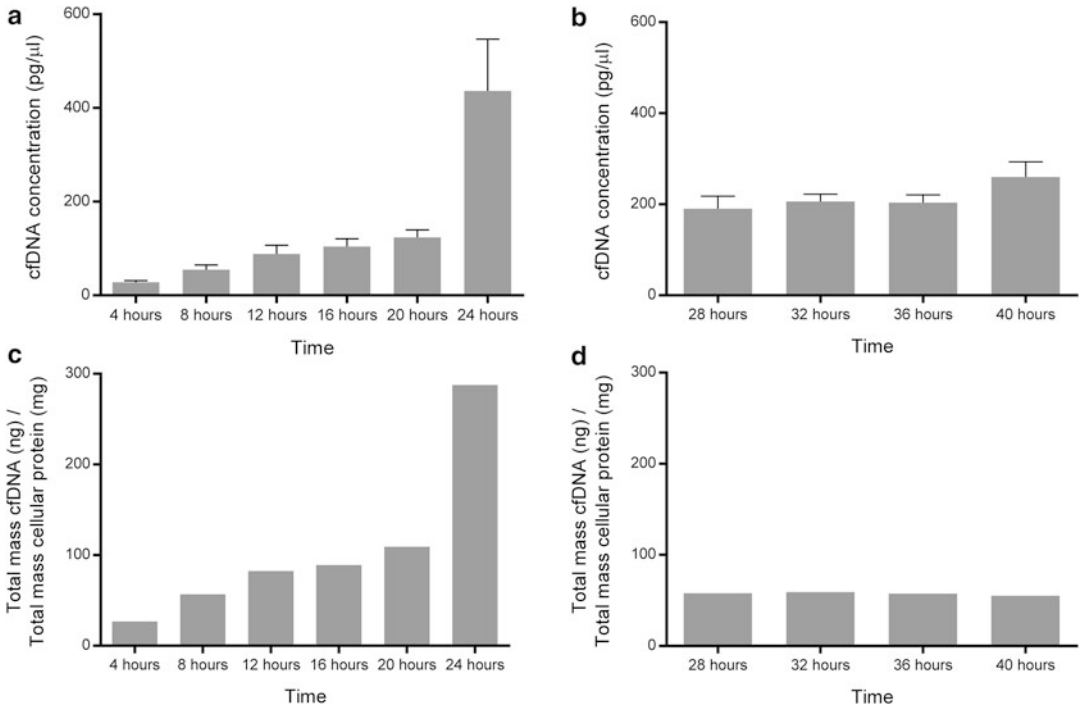

Fig. 4.1 Time-course characteristics of cfDNA released from 143 B cells. (**a**) Bar graph showing the amount of cfDNA released by 143 B cells after 4–24 h of incubation following medium renewal. (**b**) Bar graph showing the amount of cfDNA released by 143B cells after 28–40 h of incubation following medium renewal. (**c**) & (**d**) Represents the amount of cfDNA released at each time-point normalized in terms of total cellular protein

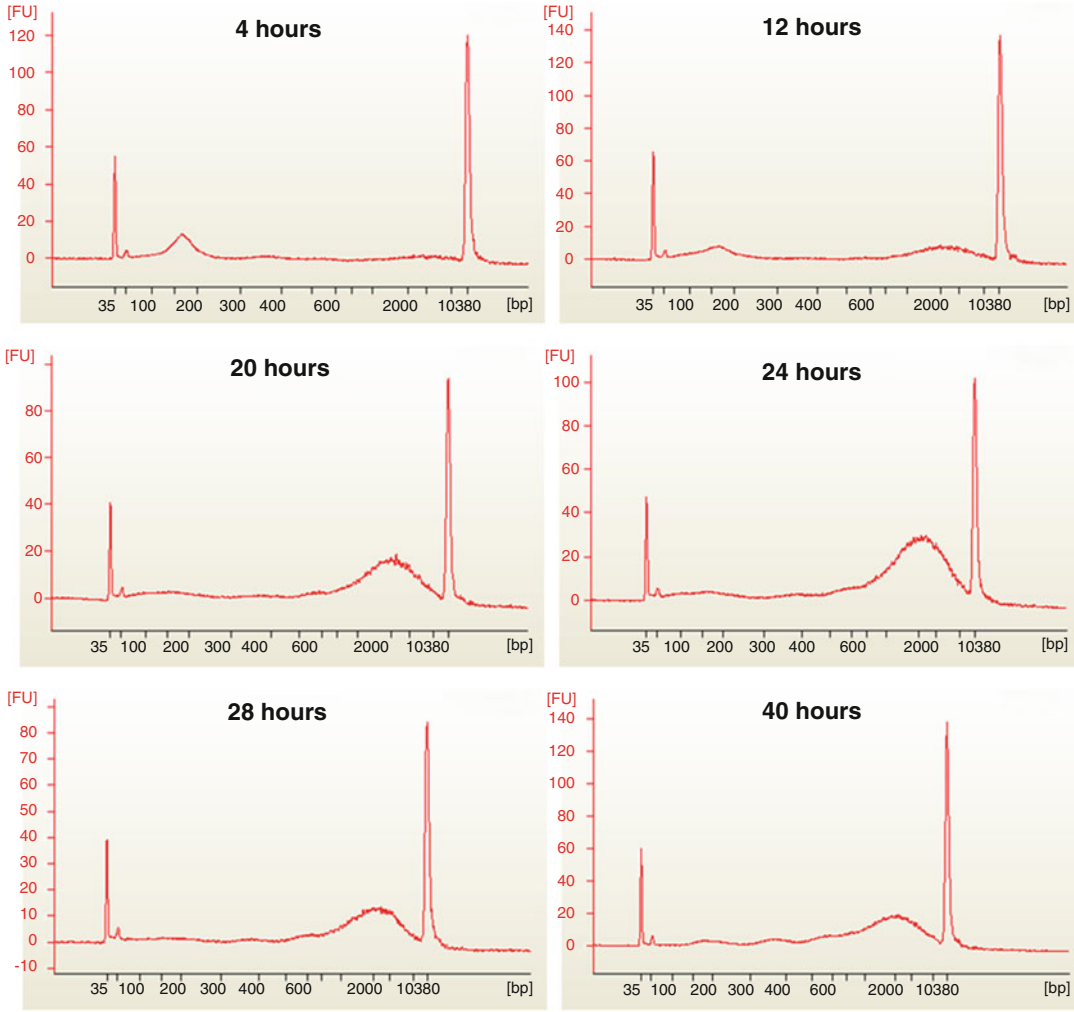

Fig. 4.2 Capillary electropherograms showing the size of cfDNA isolated after incubation at various times following medium renewal. In each electropherogram two major peaks can be seen, one at 35 bp and one at approximately 10 000 bp. These peaks correspond to the two size markers. The relative fluorescence of these markers is then used to calculate the size of the unknown samples. Thus, any deviation from the baseline, excluding the markers, indicates the size of cfDNA

suggesting an apoptotic origin. These observations demonstrate a clear correlation between an increase in the release of cfDNA and the occurrence of higher molecular weight DNA. As far as we know, this distinct size of ~2000 bp has not yet been reported. This is noteworthy, because its size suggests that it is neither from apoptotic nor necrotic origin.

To verify the observations made by electrophoretic analysis, and to help elucidate the origin of the cfDNA present at the different time points, the cells at each of the times correlating with the time-course study was analyzed for apoptosis, necrosis and cell cycle phase using flow cytometric assays. The FITC Annexin V assay revealed that a fraction of cells are apoptotic after 4 h, whereas 24 h showed almost no apoptosis or necrosis (Fig. 4.3).

Excluding the 4 h time point, these results were corroborated by the TUNEL assay, which is based on the detection of fragmented DNA (results not shown). The assay used to measure

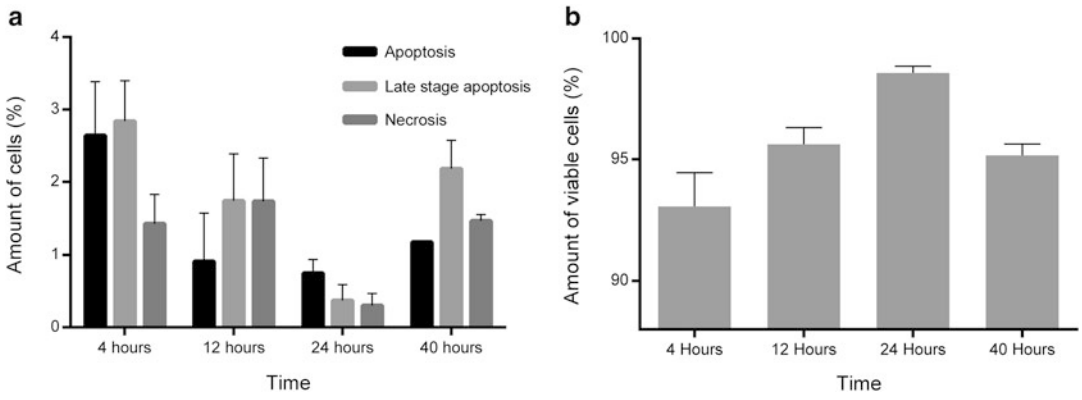

Fig. 4.3 Summary of data obtained by the FITC Annexin V flow cytometric assays. (**a**) illustrates the percentage of apoptotic, necrotic, and late-stage apoptotic 143 B cells at the different time-points. (**b**) illustrates the percentage of viable 143 B cells at each time-point. *Error bars* indicate standard deviation

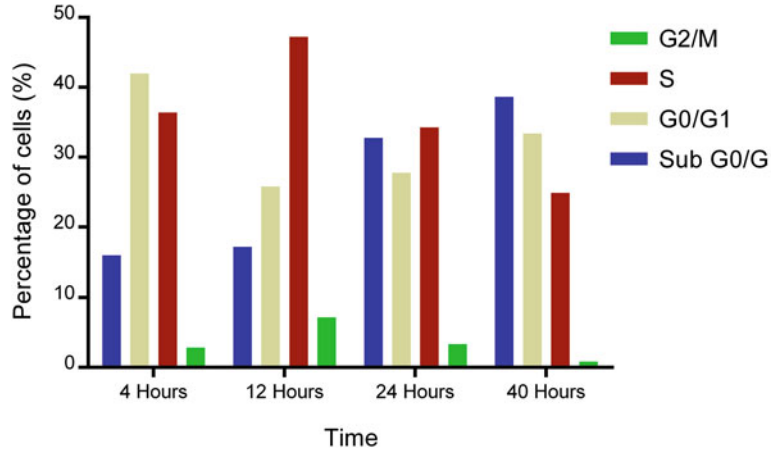

Fig. 4.4 Bar graph representing the cell cycle distribution of 143 B cells at different points in time

cell proliferation revealed that, after 24 h of growth following medium renewal, there is a decline of cells in the S phase and significant increase of cells in the sub G0/G1 phase (Fig. 4.4). This indicates that the abrupt increase of cfDNA after 24 h of incubation is not associated with the process of DNA replication.

To determine whether the cfDNA released from 143 B cells after 24 h of incubation could be similar to virtosomes, growth medium was treated with denaturing agents prior to cfDNA extraction, and were compared to untreated samples (results not shown). In all cases, the concentration of cfDNA was increased considerably by the addition of denaturing agents. These results suggest that cfDNA may be associated with proteins but whether these proteins are simply nucleosomes, lipid-protein complexes (virtosomes) or extracellular vesicles remains unclear, and requires further examination.

Conclusion

Most *in vivo* studies report that the occurrence of cfDNA is associated with apoptosis or necrosis. However, the results obtained by this study suggest that the release of cfDNA from cultured 143B cells after 24 h of incubation is not a consequence of apoptosis, necrosis or a product of DNA replication, but primarily a result of actively

released DNA, perhaps in association with a protein complex. These results also demonstrate the potential of *in vitro* cfDNA analysis to aid in the elucidation of the nature of cfDNA.

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Conflict of Interest The authors wish to declare no conflict of interest.

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5 Detection of p53 Mutations in Circulating DNA of Transplanted Hepatocellular Carcinoma Patients as a Biomarker of Tumor Recurrence

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Abstract

p53 is the most commonly mutated gene in malignant human cancers. To detect *p53* mutations in circulating DNA (cirDNA) of transplanted hepatocellular carcinoma (HCC) patients could be an interesting approach to know of any tumor recurrence. In this study, our objective was to determine the utility of this method in the diagnosis and the prognosis of HCC tumor recurrence.

Twenty four liver transplanted HCC patients were included in the study together with a group of healthy controls. Detection of the specific *p53* mutation in cirDNA was performed by high-resolution melting PCR (HRM-PCR) and COLD-PCR immediately before the transplantation. Serum anti-p53 was also determined using a p53-autoantibody ELISA kit.

The results of the HRM-PCR and COLD-PCR showed two well-differentiated groups of transplanted patients after normalization by healthy controls. These data allow us to distinguish between patients with *p53* mutated cirDNA and those with wild type cirDNA. Moreover, we have

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found that most of *p53* mutated patients also presented elevated anti-p53 antibodies. The present results indicate that it is possible to detect mutated *p53* genes with the cirDNA and that this could be used as a biomarker of tumor recurrence during the clinical evolution of the transplanted patients.

Keywords
Circulating DNA • *p53* mutation • Anti-p53 antibodies • Hepatocellular carcinoma

Introduction

p53 is the most commonly mutated gene in human cancers including hepatocellular carcinoma (HCC) (Bernard et al. 2014). Both the mutated p53 protein and anti-p53 antibodies are related to the clinical characteristics, diagnosis and therapeutic response of HCC (Akere and Otegbayo 2007). Effective methods for the treatment of liver cancer include liver transplantation. Because tumor recurrence is the major obstacle to improved prognosis after this therapeutic option, novel tumor biomarkers should be considered. The use of cirDNA as a tool for evaluation of *p53* mutation in the patients selected for liver transplantation may be an interesting approach (Macher et al. 2012). Transplanted organ cells suffering damage liberate DNA. Thus, during organ rejection, apoptotic cell death leads to the release of specific transplanted organ DNA into the host plasma.

The objective of this study was to determine the utility of detecting *p53* mutations in cirDNA as a biomarker of tumor recurrence in transplanted HCC patients.

Materials and Methods

Serum cirDNA from 24 patients with HCC preceding liver transplantation were analyzed. We studied the exons 6, 7, and 8 from the *p53* gene in order to find different plots compared to the wild type (wt) controls. Detection of these exons in cirDNA was performed by high resolution melting PCR (HRM-PCR) and COLD-PCR immediately before the transplantation. Results were normalized with two wt controls.

In addition, serum anti-p53 was measured using a p53-autoantibody ELISA kit in TRITURUS.

Results

p53 Mutations in HCC

We analyzed exon 6 (Fig. 5.1), exon 7 (Fig. 5.2) and exon 8 (Fig. 5.3) and found different plots that were classified into 3 groups: group 0 (patients grouped with wt); group 1 (patients with some differences with wt group); group 2 (very different to wt group).

Serum Anti-p53

Table 5.1 shows the levels of anti-p53 antibodies. We found a high number of patients with genetic variants in exon 8 with elevated levels of anti-p53 antibodies. However, low levels of anti-p53 did not involve those patients grouped with wt controls. Moreover, analysis of exons 6 and 7 showed a high number of patients differing from the wild control that was not associated with anti-p53 levels.

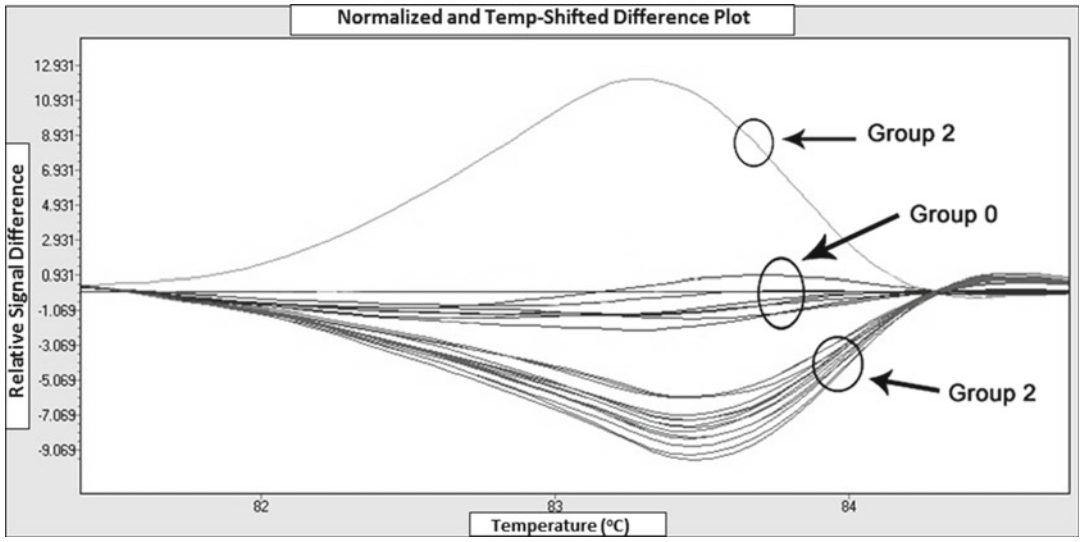

Fig. 5.1 Exon 6 analysis showing the groups 0 and 2

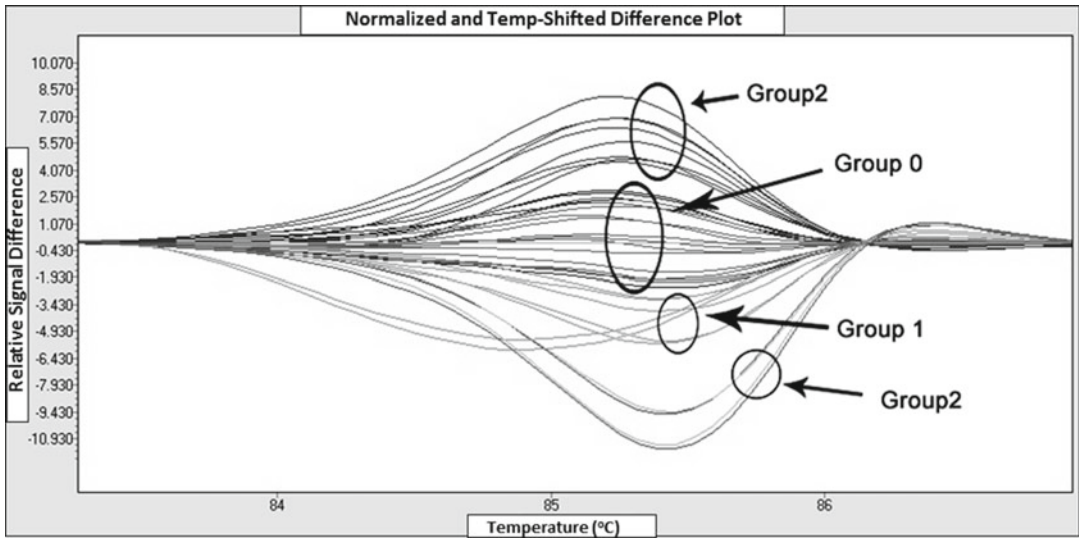

Fig. 5.2 Exon 7 analysis showing the groups 0, 1 and 2

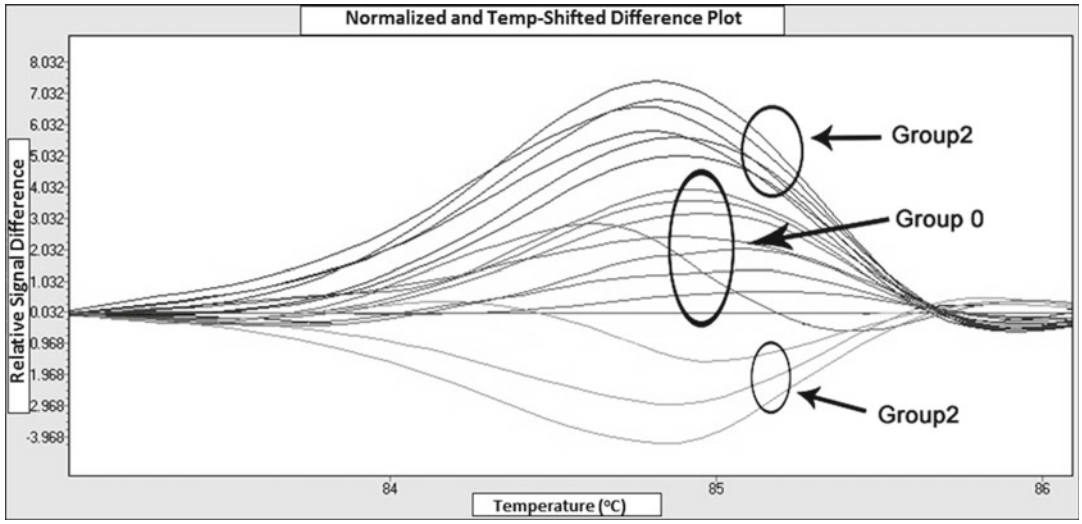

Fig. 5.3 Exon 8 analysis showing the groups 0 and 2

Table 5.1 Anti-p53 antibody levels per groups 0–2 and per exon

Ac anti P-53 (U/µl)	Exon 6 Wt	Exon 6 Wt		Exon 7 Wt	Exon 7 Wt		Exon 8 Wt	Exon 8 Wt	
		Group 1	Group 2		Group 1	Group 2		Group 1	Group 2
>0.15..... (n=8)	0	7	1	3	4	1	1	1	6
0.1-0.15..... (n=2)	1	1	0	1	0	1	1	1	0
<0.1..... (n=14)	2	4	8	5	1	8	6	2	6

Discussion

The present results indicate that it is possible to detect genetic variants with respect to the wt *p53* gene in the cirDNA. Analysis of number of exons different from the wt in each patient showed that only one patient was grouped with the wt for all exons analyzed. Moreover, most of the patients had either two (46%) or three (38%) exons with genetic variants with respect to the wt control, independently of anti-p53 levels.

In conclusion, this method is an easy and inexpensive manner of monitoring the *p53* gene in cirDNA. In this way, it could be used as a biomarker of tumor recurrence after transplantation.

Conflict of Interest No conflict of interest.

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6

Unbiased Detection of Somatic Copy Number Aberrations in cfDNA of Lung Cancer Cases and High-Risk Controls with Low Coverage Whole Genome Sequencing

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Abstract

Molecular profiling using low coverage whole genome sequencing of cell free DNA (cfDNA) represents a non-targeted approach to identify multiple somatic copy number alterations (SCNA) across different lung cancer subtypes. We aim to establish that SCNA can be detected in cfDNA of lung cancer cases.

Standard protocols were followed to process matched cfDNA, formalin-fixed paraffin embedded (FFPE) tumour and lymphocyte DNA. Copy number profiles for cfDNA or FFPE DNA were normalised to profiles from matched lymphocyte DNA with the software CNAnorm. Technical sensitivity was determined by spiking different proportions of FFPE tumour DNA into cfDNA from controls.

The median genome coverage was 0.26X (range 0.05X–0.97X). For two advanced stage cases there was a positive correlation between copy number ratio profiles of matched cfDNA and FFPE DNA ($r=0.62$, $p<0.0001$ and $r=0.75$, $p<0.0001$). There was no correlation for four advanced and two early stage cases. There were low magnitude copy number aberrations detected in high-risk controls ($N=5$). We detected spiked FFPE DNA derived SCNAs with a tumour fraction as low as 10 % of cfDNA.

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Our preliminary results demonstrate non-invasive detection of tumour-derived copy number alterations in advanced lung cancer cases with low coverage whole genome sequencing. Clinical characteristics and treatment may influence whether SCNA are detected in cfDNA.

Keywords
Cell-free DNA • Low coverage • Whole genome • Sequencing • Lung cancer

Introduction

Lung cancer is the most common cause of cancer death worldwide (Lozano et al. 2010). Most people with lung cancer are diagnosed with advanced disease that is not amenable to curative treatment and the 1 year survival rate is 16 % (Walters et al. 2013). Improving the early detection of lung cancer could lead to fewer deaths because more patients could have curative treatment. Importantly, such a test should detect both lung cancer subtypes, small cell (SCLC) and non-small cell (NSCLC).

We aim to establish whether somatic copy number aberrations (SCNA) can be detected in cell free DNA (cfDNA) of lung cancer patients with early and late stage disease, with low coverage whole genome sequencing (WGS). We hypothesise that the number and magnitude of SCNA might also serve as a discriminative test to aid early lung cancer detection.

Materials and Methods

Blood and tumour samples from lung cancer cases and controls were collected in the Sheffield ResoLuCENT study (https://clinicaltrials.gov/ct2/show/NCT00693836) For cfDNA, blood was collected in EDTA tubes and processed by centrifugation at 800 g for 10 mins, then 1600 g for 10 mins, within 2 h of withdrawal. Plasma was stored at −80 °C and once thawed, was centrifuged at 1000 g for 5 min before use. CfDNA was extracted from 2–3mls of plasma with the Circulating Nucleic Acid Kit (Qiagen) and quantified by measuring GAPDH with SYBR green PCR in comparison to a standard curve with $R^2 > 0.99$. Lymphocyte DNA was extracted by an automated magnetic bead method (Chemagen) and formalin-fixed paraffin embedded (FFPE) tumour DNA was extracted with the FFPE DNA kit (Qiagen). Both were quantified by the Qubit™ 2.0 fluorometer.

DNA libraries were prepared with the NEBnext ultra DNA kit (New England Biolabs). Input DNA for cfDNA was 6–20 ng and for FFPE or lymphocyte DNA 100 ng of fragmented DNA. The quality and quantity of the libraries were assessed using the Agilent Tapestation. Libraries were pooled for Illumina paired-end low coverage whole genome sequencing using the Illumina MiSeq (2 × 150bp reads) or Hiseq (2 × 100bp reads) systems. Analysis parameters in the software package CNAnorm (Wood et al. 2010) were adjusted for sample heterogeneity. Profiles were normalized to genomic DNA to establish copy number ratios and FFPE DNA and cfDNA ratios were correlated to establish the presence of tumour derived SCNA in cfDNA.

Results

Thirteen samples from cases (N = 8) and controls (N = 5) were sequenced and the mean total number of reads for each sample was 14,605,710. Of these, 89 % mapped to the human reference genome (hg19) after filtering. The median genome coverage was 0.26X (range 0.05X–0.97X). For two advanced stage cases with squamous and small cell lung cancer there was a significant positive correlation between the

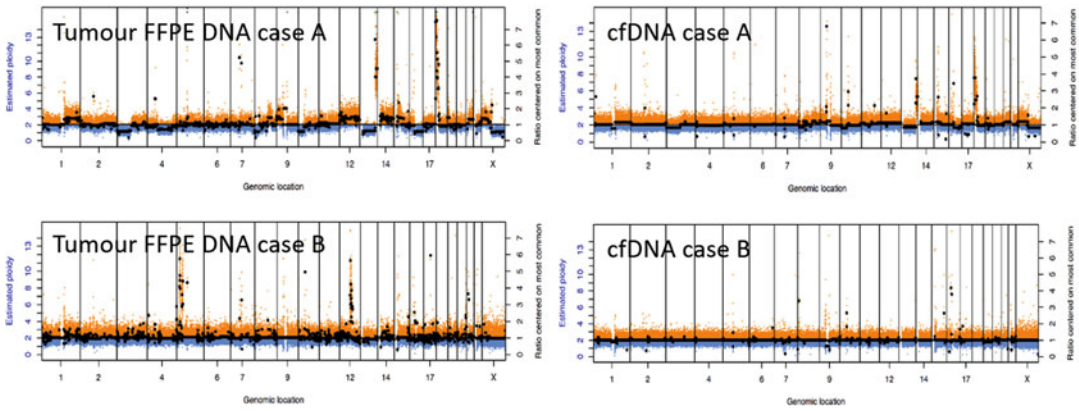

Fig. 6.1 Tumour FFPE DNA and cfDNA copy number profiles are displayed to demonstrate examples of the identification of SCNA in cfDNA with a similar profile to the FFPE DNA and high correlation (r = 0.75) (Case A) and a case where there were no similarities between the profiles of the FFPE DNA and cfDNA with no correlation (r = −0.29) (Case B). Each *dot* represents a 'window' and *black line* or *segment* represents consecutive windows of similar ratio. The colour *orange* show copy number gains and *blue* shows copy number losses

copy number ratio profiles of matched cfDNA and FFPE DNA (Spearman Rank $r = 0.62$, $r = 0.75$ respectively, $p < 0.0001$). However, there was no correlation for four advanced and two untreated early stage cases. Of the four advanced cases, two with adenocarcinoma had stable or low volume disease, one had no extra-cranial disease, and one had small cell lung cancer (SCLC) that had been treated with one cycle of chemotherapy (Fig. 6.1).

There were variable frequency, but low magnitude copy number aberrations detected in heavy smoking high risk controls (N = 5) that had a risk of lung cancer of ≥5 % over 5 years, according to the Liverpool Lung Project Risk model (Cassidy et al. 2008).

The estimated tumour content identified in cfDNA from cases varied from 2.5 to 8.0 % (N = 6). In comparison, we detected spiked FFPE DNA derived SCNAs with a tumour fraction as low as 10 % of cfDNA from healthy volunteers.

Discussion

Our preliminary results demonstrate the non-invasive detection of tumour derived copy number alterations in advanced lung cancer cases with low coverage whole genome sequencing. Clinical characteristics, histology and treatment may influence SCNA detection in cfDNA. It is well described in the literature that lower allele fractions of cell free tumor DNA are found in lung cancer cases with early compared to late stage disease (Bettegowda et al. 2014) and allele fractions decline with both targeted therapy (Piotrowska et al. 2015) and chemotherapy (Tie et al. 2015). To further optimise this approach we will focus on untreated advanced cases. To identify SCNA in cfDNA of early stage cases we will increase sequencing coverage and input cfDNA quantity. A targeted sequencing approach may increase test sensitivity for the detection of SCNA but at the potential cost of missing important aberrations across different histological subtypes.

The significance of the detection of copy number aberrations in high-risk controls is unknown. Izumchenko et al. (2015) recently detected mutations in cfDNA by digital droplet PCR that matched mutations in pre-invasive bronchial lesions with allele fractions <1 %. No control in our cohort has since developed lung cancer with a median follow up of 5.0 years (range 3.4–9.5 years).

The establishment of a robust genomic instability score will be used to determine whether SCNA can distinguish between high-risk controls

and early lung cancer cases for both NSCLC and SCLC subtypes.

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Conflicts of Interest No author has a conflict of interest to declare.

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Liquid Profiling in Lung Cancer – Quantification of Extracellular miRNAs in Bronchial Lavage

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Abstract

Extracellular miRNAs cannot only be isolated from different body fluids like plasma and serum, but also from bronchial lavage samples (BL) obtained by bronchoscopy. Alterations in the expression of microRNAs might be useful for a discrimination of lung cancer patients from patients with a benign lung disease. We profiled extracellular microRNAs from three BL pools of lung cancer patients and three BL pools from a control group (patients with a benign lung disease) with TaqMan MicroRNA Array cards. For the confirmation of these results, we analyzed a panel of eight miRNAs in a qRT-PCR of the BL of 30 different lung cancer and non-cancerous patients. For the data normalization, we used exogenously added cel-miR-39 RNA. Using microRNA arrays, we found a panel of eight microRNAs (hsa-miR 19b-1, 1285, 1289, 1303, 217, 29a-5p, 548-3p, 650) that were differentially expressed between the lung cancer and the non-cancerous group. Further investigation by qPCR revealed five microRNAs (U6 snRNA, hsa-miR 1285, 1303, 29a-5p, 650) that were significantly up-regulated in patients with lung cancer. In bronchial lavage samples, the five microRNAs identified in this study may have a diagnostic potential to be used as biomarkers in lung cancer.

Keywords

Bronchial lavage • miRNA • Real-time PCR • Lung cancer • Detection

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Introduction

Patients with lung cancer often develop disease associated symptoms at an advanced stage when the treatment options are only palliative and the survival time rather limited (Malvezzi et al. 2013). The large National Lung Screening Trial (NLST) demonstrated that a low-dose CT (LDCT) screening can reduce the mortality by 20 % as compared to chest X-ray examination (Aberle et al. 2011). Unfortunately, the high number of false positive results is one of the reasons that this method is not yet the method of choice for the detection of lung cancer at an early stage. Additionally, the examination of a tissue sample obtained by an invasive procedure is necessary for a definitive diagnosis. Thus, the combination of LDCT with clinical meaningful biomarkers might not only lead to a test with improved sensitivity, but to a diagnostic procedure which is more straightforward than the standard system in practice so far. Biomarkers that are potentially eligible for such an application could be miRNAs because they are stable and easy to detect in different biological fluids and washings (Jeong 2014; Ulivi and Zoli 2014).

These microRNAs are small non-coding RNAs that regulate post-translational protein expression and thereby have an influence on a wide range of biological functions such as cellular proliferation, differentiation and apoptosis (Lewis et al. 2005). Their expression seems to be specifically deregulated in the development of cancer and able to alter the expression of tumor-suppressor genes and oncogenes (Kong et al. 2012). Alterations in microRNA expression have been shown for several solid tumors including cancer of the lung (Lin et al. 2010). Almost all of the published reports on a differential miRNA expression pattern in lung cancer patients used plasma or serum as starting material. As part of the diagnostic workup for patients with suspected lung cancer, a bronchoscopy is routinely performed and frequently bronchial lavage fluid (BL) is obtained. Assuming that such a bronchial washing is closer to the "place of action" than plasma or serum, we hypothesized that an examination of this liquid would be better suited for the detection of a lung cancer associated miRNA signature.

Materials and Methods

Patients

All 60 patients underwent bronchoscopic examination for suspected lung cancer. During bronchoscopy 10–20 mL of isotonic saline solution were instilled and collected by aspiration with a flexible bronchoscope from the region of the suspicious lesion. Bronchial lavage samples were centrifuged and stored at −80 °C. We used bronchial lavage samples from 30 patients with benign lung diseases (17 male and 13 female) with a median age of 63.5 and 30 bronchial lavage samples from patients with lung cancer (21 male and 9 female) with a median age of 64.5. Patients with benign lung diseases were suffering from fibrosis, pneumonia, chronic obstructive pulmonary disease (COPD) or tracheal stenosis while lung cancer patients were diagnosed with squamous cell lung cancer or adenocarcinoma of the lung in an advanced stage.

RNA Isolation and Quantitative RT-PCR

The RNA was isolated from 1 mL bronchial lavage samples obtained from patients with lung cancer and a benign lung disease, respectively, using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the normalization synthetic *Caenorhabditis elegans* microRNA cel-miR 39 (Qiagen, Hilden, Germany) was added. The total RNA concentration was quantified by absorbance at 260 nm using a Nanodrop™1000 spectrophotometer. The quantity of selected microRNAs were assessed using specific TaqMan microRNA Assays. Reverse transcription was performed according to the manufacturers. Each cDNA was pre-amplified in 12 cycles using the TaqMan PreAmp Master Mix and the specific TaqMan®

MicroRNA Assays. For real-time qPCR,1.6 µL of pre-amplified product was used in a total volume of 20 µL reaction mix. Additionally the reaction contained the specific TaqMan® MicroRNA Assay and the TaqMan® UniversalMasterMix II without UNG. The Ct value of the specific microRNA was determined relative to the corresponding Ct value of the added exogenous control gene cel-miR 39 resulting in the ΔCt value that was used for relative quantification of microRNA expression. Based on previous results, we selected a panel of 5 miRNAs (U6 snRNA, hsa-miR 1285, 1303, 29a-5p, 650) that were significantly up-regulated in patients with lung cancer.

Results

When we examined the microRNA expression pattern from three different bronchial lavage pools of lung cancer and non-cancerous patients, each using the TaqMan MicroRNA Array cards, eight microRNAs were consistently up- or down-regulated in the lung cancer samples (data not shown). In addition, the expression of U6 snRNA was relatively constant on the cards. As a next step we tried to validate the array card data by single-plex qRT-PCR on single lavage samples. In contrast to the microRNA Array cards, there was a difference in the expression of U6 when this miRNA was examined by single-plex qRT-PCR. Therefore, this miRNA was not useful as a reference gene for data normalization. Instead we applied the exogenously added cel-mir 39 for normalization. With the qRT-PCR we found U6 snRNA ($p=0.001$), hsa-miR 1285 ($p=0.005$), hsa-miR 1303 ($p=0.008$), hsa-miR 29a-5p ($p=0.014$) and hsa-miR 650 ($p=0.027$) significantly up-regulated in bronchial lavage samples from lung cancer patients. The other four investigated microRNAs (hsa-miR 19b-1, 1289, 217 and 548-3p) showed a trend to be up-regulated, but the results were not significant (Fig. 7.1). Interestingly, we did not find any microRNAs down-regulated in bronchial lavage samples of lung cancer patients as compared to the control group.

Discussion

The aim of this paper was to analyze the miRNA expression pattern in bronchial lavage samples in order to discriminate lung cancer patients from a control group. In a first step, a pool of total RNA from BL of lung cancer patients and a control group was reverse transcribed and the cDNA analyzed with microRNA Array cards. A panel of eight miRNAs that were differently expressed was chosen for further analysis by single-plex qPCR. We observed that the use of 100 ng total RNA for one single-plex reaction as recommended by the company plus a pre-amplification step is necessary to get reliable and reproducible results. We identified a set of five microRNAs (U6 snRNA, hsa-miR 1285, 1303, 29a-5p, 650) that were significantly up-regulated in patients with lung cancer. None of these microRNAs have been described before as being over-expressed in lung cancer patients.

Almost all previous studies concerning extracellular miRNA expression profiles in lung cancer patients used plasma or serum (Chen et al. 2014; Ramshankar and Krishnamurthy 2013). In contrast, we decided to use BL as the starting material, assuming that a microRNA signature from this liquid might be more specific than plasma or serum to detect changes in the diseased lung. In fact, it was earlier demonstrated that the miRNA expression pattern from plasma and BL is different (Molina-Pinelo et al. 2012; Rodriguez et al. 2014). The microRNA expression pattern obtained in this work from lung cancer patients has not been described before. So far, there was only one paper published in which miRNA from the BL of lung cancer patients was analyzed (Kim et al. 2015). Unfortunately, there is no concordance between these results and ours. The reasons for this difference could be due to a different sample preparation, different methods for the RNA isolation and the fact that Kim et al. used miR-U6 as a reference gene for data normalization. In contrast, we found a different expression of miR-U6 in the BL in cancer patients as compared to the control group and therefore excluded U6 as a standard. Nevertheless, these discordant data between

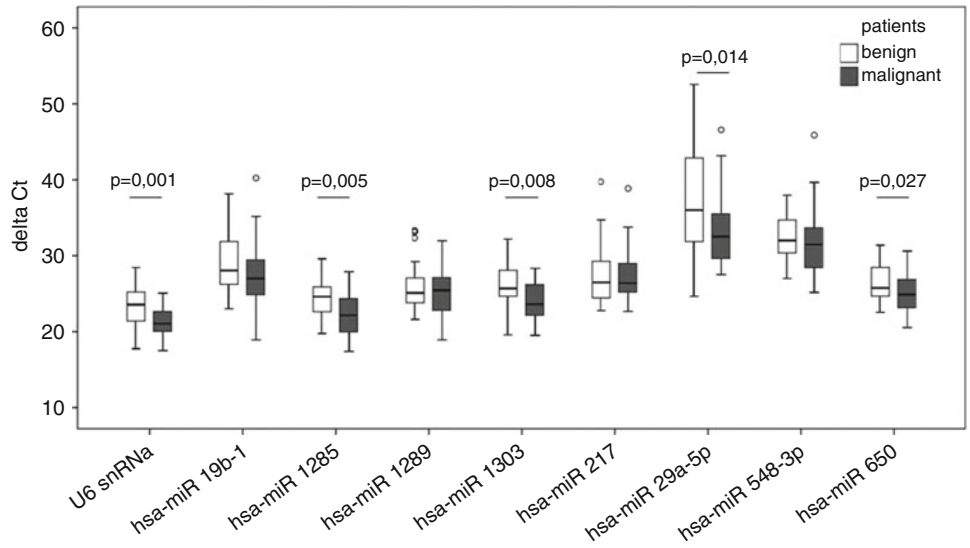

Fig. 7.1 Quantitative RT-PCR of microRNA showed a signature of five microRNAs to be significantly up-regulated in bronchial lavage samples of lung cancer patients compared to non-cancerous patients

Kim et al. and our results is a general reflection of the many discordant reports published on the miRNA expression patterns in plasma and serum. One of the reasons for this discordance is the fact that pre-analytic sample preparation methods are different and that there is no standard so far. Additionally we can expect that different RNA isolation protocols, the use of different platforms for the miRNA quantification (Mestdagh et al. 2014) and the method for data normalization have a strong influence on the results. Even if our data demonstrate the ability to use miRNA expression patterns as a means for differentiating lung cancer patients from a control population, only a standardization of the complete workflow will yield comparable and clinically meaningful results.

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Conflict of Interest The authors do not report a conflict of interest.

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8 Screening of KRAS Mutation in Pre- and Post-Surgery Serum of Patients Suffering from Colon Cancer by COLD-PCR HRM

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Abstract

Genomic characterization of cell-free circulating tumour DNA (ctDNA) may offer an opportunity to assess clonal dynamics throughout the course of a patient's illness. The existence of KRAS driver mutations in colon cancer patients is determinant to decide their treatment and to predict their outcome. DNA is extracted automatically from 400 μL of serum using the MagNa Pure Compact with the Nucleic Acid Isolation Kit I. DNA amplification, COLD-PCR and HRM were performed in the same run in the Light Cycler 480.

We found three different situations: pre- and post-surgical samples grouped with the negative control, pre-surgical samples appear to group with the positive control and the post-surgical samples appear to group with the negative control and finally both samples, pre- and post-surgical ones, appear to be grouped with the positive control. COLD-PCR HRM is a cost-effective way for screening one of the most common driver mutations to predict the worst prognosis in colorectal cancer.

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Keywords
KRAS mutation • Colorectal cancer • cf-DNA • Genomic signature

Introduction

Tumor heterogeneity has a challenge for personalized cancer medicine since a single needle biopsy or surgical excision is unlikely to accurately capture the complete genomic landscape of a patient's cancer (Diehl et al. 2008). Genomic characterization of cell-free circulating tumour DNA (ctDNA) may offer an opportunity to assess clonal dynamics throughout the course of a patient's illness (Kidess et al. 2015). The existence of KRAS driver mutations in colon cancer patients is a determinant in deciding their treatment and to predict their outcome (Bettegowda et al. 2014). The aim of this study was to discriminate, in a inexpensive way, between patients with mutations in the KRAS gene in serum cell-free DNA (cf-DNA) by Cold High resolution melting PCR (COLD-PCR HRM).

Materials and Methods

Patients included in the study were previously diagnosed with colorectal cancer, but it is not known if they carry some of the most common KRAS mutations. The positive control is DNA from a cell line with one of the most common mutations in colorectal cancer in the KRAS gene (c.35G>T), negative controls are patients with the wild type KRAS gene (diagnosed by the Department of Pathology) and healthy volunteers.

One tube of 10 mL serum is obtained from each patient before surgical resection and another 24 h after surgery. DNA is extracted automatically from 400 μL of serum using the MagNa Pure Compact (Roche Diagnostics, Basel, Switzerland), with the Nucleic Acid Isolation Kit I. DNA amplification, COLD-PCR and HRM were performed in the same run in the Light Cycler 480. The interpretation of the results is performed with the Gene Scanning software from Light Cycler 480 (Roche Diagnostics).

Results

After normalizing the melting curves and calculating the different plots by subtracting the profile controls, we found three different situations:

(a) Pre- and post-surgical samples grouped with negative control after the Gene Scanning Module in the LightCycler® 480 Software processed the raw melting curve data (Fig. 8.1).
(b) Pre-surgical sample appeared to be grouped with the positive control and the post-surgical appears with the negative control (Fig. 8.2).

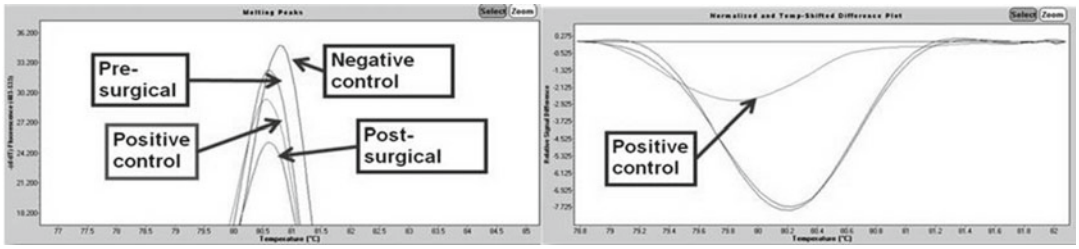

Fig. 8.1 Pre- and post-surgical samples group with the negative control

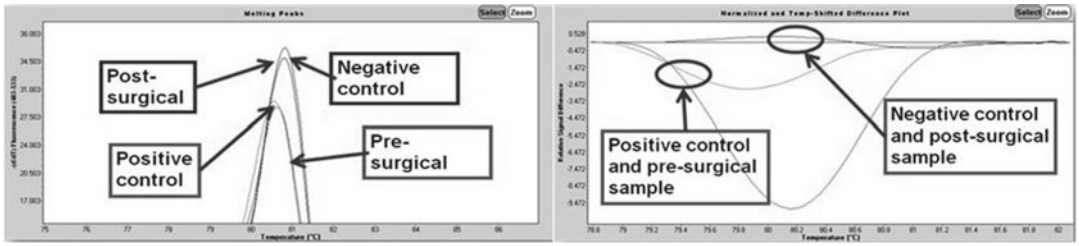

Fig. 8.2 Pre-surgical sample appears grouped with the positive control and the post-surgical sample appears with the negative control

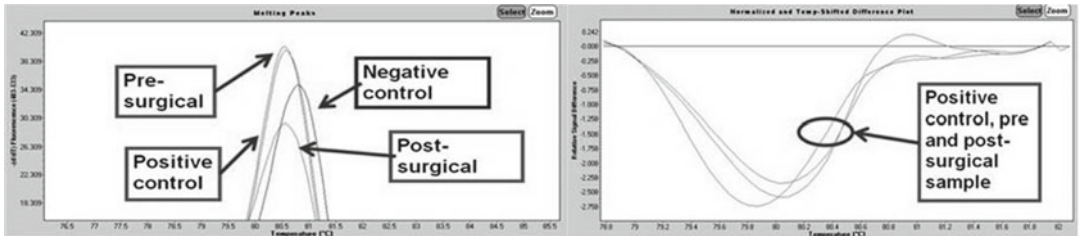

Fig. 8.3 Pre- and post-surgical samples appear to be grouped with the positive control

(c) Both samples, pre- and post-surgical ones, appear to be grouped with the positive control (Fig. 8.3).

In the first situation it should not be necessary to analyse the KRAS gene in the biopsy because the results show that any copy of the mutation existed after surgery resection. In the second case (b) sequencing the KRAS of the biopsy should be necessary to confirm the mutation. And finally, in the third case (c), metastasis should be considered and more exhaustive monitoring is also necessary.

Discussion

The results show that the slope of the melting curve of patients with one of the KRAS mutations is homogeneous (grouped with positive control) and different from healthy controls. COLD-PCR HRM is a cost-effective way for screening one of the most common driver mutations to predict the worst prognosis in colorectal cancer.

Conflict of Interest None.

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Non-dividing Cell Virtosomes Affect *In Vitro* and *In Vivo* Tumour Cell Replication

9

Mariano Garcia-Arranz, Damian Garcia-Olmo, Luz Vega-Clemente, Maurice Stroun, and Peter B. Gahan

Abstract

In vitro studies of partially purified virtosomes from rat liver showed inhibition of cell multiplication in four normal and two tumour cell lines. *In vivo*, the liver virtosomes slowed tumour growth and limited metastases in rats bearing DHD/K12-PROb cell initiated tumours.

Keywords

Tumour cells • Virtosomes • Tumour development • Metastases

Introduction

Virtosomes are comprised of a DNA-RNA-lipoprotein complex. They are released spontaneously from healthy human, other mammalian, avian, amphibian and plant cells in a regulated and energy-dependent manner (Gahan and Stroun 2010). The released virtosomes have been demonstrated to enter other cells (Anker et al. 1980, 1994; Adams et al. 1997). If the recipient cells are of a different cell type to those cells from which the virtosomes emanated, then the biology of the recipient cells may be modified. Thus, the uptake of virtosomes from either non-dividing mouse lymphocytes or hepatocytes by mouse tumour cell lines J477 and P497 cells results in the blocking of DNA synthesis in the recipient cells (Adams et al. 1997).

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The present study concerns an extension of the previous *in vitro* virtosome release and uptake studies. Hence, the effects of virtosomes isolated from rat liver, comprised essentially of non-dividing cell populations, was tested on four established, actively-dividing healthy cell lines and two tumour cell lines.

An *in vivo* study determined the effect of liver virtosomes on the growth and metastases of tumours induced with DHD/K12-PROb cells in 6 weeks old male BIDX rats.

Materials and Methods

Rat liver virtosomes were freshly prepared by homogenisation and centrifugation by a modified method of Adams et al. (1997) and the final supernatant containing the virtosomes was sterilised by filtration (0.22 μm) in a laminar flow cabinet and stored at 4 °C until use.

In vitro experiments were performed on smooth muscle cells, human umbilical vein endothelial cells, human fibrobasts, mouse embryonic fibroblasts and two tumour cell lines namely, human Duke's type B colorectal adenocarcinoma cells and human connective tissue fibrosarcoma cells.

Cultures were seeded and left for 2–4 days to stabilize prior to the addition of the virtosomes at varying concentrations – full extract and series of dilutions from 75 to 0,5 %. Culture medium + virtosomes was renewed every 24 h as was the culture medium – virtosomes for the control cultures and cells counted at 24 h time intervals using a Coulter counter Micro Count 1100. Cell death was monitored using Trypan blue staining.

In vivo experiments were performed with 6-weeks old male syngenic BIDX rats and tumours initiated by subcutaneous injection of 1.0×10^6 DHD/K12-PROb cells in 0.25 ml PBS. One ml of virtosomal extract was injected via a tail vein after withdrawal of 1.0 ml blood.

A range of tissues were removed for histological analysis.

Results

In vitro experiments The continuous presence of varying virtosomal concentrations (100–10 %) gave almost complete cell multiplication inhibition of both tumour and normal cell lines. Significant inhibition resulted on dilution to 2 %. The results were not due to cell death.

In vivo experiments Control rats injected either with saline or with 10 consecutive daily injections of partially-purified virtosomes at day one or with an additional 10 consecutive daily injections 3 weeks later showed no ill effects. Rats with induced tumours + 10 daily virtosomal injections showed a reduction in tumour size up to 2 months later and no liver and colon metastases occurred. Rats receiving a second series of virtosomes 4 weeks after the first series showed little response re primary tumour growth.

Histological analysis of a range of tissues, including the spleen, showed no evidence of an immune reaction resulting from the injection of the protein present in the preparation.

Discussion

The addition of various concentrations (2.0–100 %) of cytosolic virtosomes from non-dividing hepatic cells to the cell cultures resulted in either the partial or almost total inhibition of cell multiplication, confirming earlier studies on normal and tumour cells (Adams et al 1997).

Preliminary examination of anti-tumour activity *in vivo* showed that a single daily dose

for a 10-day period after tumour initiation resulted in a reduction in tumour size both at 3 weeks and at 2 months. However, the second 10-day series of virtosome injections given 1 month later was far less effective. The virtosomal effect in blocking cell multiplication is transient in both *in vitro* and *in vivo* studies, resulting in a release from inhibition at times after inhibition initiation. These results could indicate the possibility of a response due to the initiation of an immune reaction. Such an event would be due to the presence of proteins in the injected preparation rather than DNA since the latter is complexed with lipoprotein. However, the syngenic animals used in the experiment together with the results obtained from the histological analysis of the various tissues examined do not support this explanation for the tumour inhibition.

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Features of Circulating DNA Fragmentation in Blood of Healthy Females and Breast Cancer Patients

10

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Abstract

Size and termini of cell-free DNA molecules circulating in blood plasma and being bound with blood cell surface of healthy females and untreated breast cancer patients were investigated. The size and concentration of circulating blood DNA were analyzed by Agilent 2100 Bioanalyser ™ and TaqMan PCR. The termini of circulating DNA were examined by ligation using biotinylated double-stranded oligonucleotide adapters with random 1–3 b overhangs of both chains and subsequent quantification by PCR. Short (180 bp) and longer (>8 kbp) DNA fragments were found in cell free DNA from both groups, but short were less represented in primary breast cancer patient plasma. Predominantly high molecular weight DNA was found in cell surface bound DNA both in healthy females and breast cancer patients with a minor fraction of short fragments. Heterogeneous DNA molecules with diverse 5′- and 3′- protruding as well as blunt ends were found both in plasma DNA and cell bound DNA from healthy individuals. Cell surface bound DNA from breast cancer patients mainly contains 5′-protruding ends, whereas 5′- and 3′-protruding ends

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are equally presented in cell free DNA from these patients. The data obtained obviously reflect over-representation of specific nucleases in breast cancer.

Keywords
Circulating DNA • Termini • Size • Length • Cancer

Introduction

It is considered that circulating DNA (cirDNA) is represented by short fragments of mainly genomic DNA circulating as double-stranded molecules composed of nucleosomes. Concentration and fragmentation of cirDNA were shown to differ in the healthy state, trauma, cancer, autoimmune and infectious diseases (Bryzgunova and Laktionov 2014). DNA hydrolysis is a basic process of cirDNA generation beginning from DNA degradation by caspases during apoptosis and then continuing through the action of cellular nucleases and nucleases circulating in blood (Bryzgunova and Laktionov 2014). Thus, DNA hydrolysis determines DNA fragmentation and the structure of the DNA ends. It is known that activity of DNA hydrolyzing enzymes differ in cancer patients and thus in this study, we analyzed the size and termini of cirDNA in blood of healthy females (HFs) and patients with primary breast cancer (BCPs) (T1-2N0M0).

Materials and Methods

Blood samples of previously untreated BCPs (n = 18, T1-2N0M0) and HFs (n = 50) were enrolled in the study in accordance with current rules. Cell-free DNA (cfDNA) and cell surface bound DNA (csbDNA) were obtained and isolated as described earlier (Tamkovich et al. 2005).

For investigation of DNA fragmentation, DNA from normal blood samples (n = 25) were polled, DNAs from all BCP blood samples (n = 8) were analyzed individually. CirDNA were separated by 6% PAAG, short fragments (100–1000 bp) were isolated by electro-elution. The efficacy of short DNA isolation was confirmed by isolation from the gel of 1 ng and 40 pg of PCR product (194 bp). Total cirDNA and short cirDNA were quantified by TaqMan PCR for L1 repeats (Bryzgunova et al. 2011) and an Agilent 2100 Bioanalyser ™ using High Sensitivity DNA Kit was used to characterize the size distribution of cirDNA.

To examine cirDNA termini, cfDNA and csbDNA from HFs blood (n = 25) and blood of BCPs (n = 10) were isolated (Tamkovich et al. 2005) and concentrated (Bryzgunova et al. 2011). Pooled DNA samples from either normal or cancer blood were incubated with Kinase (Fermentas) for 1 h at 37 °C in exchange buffer. Phosphorylated DNA was then isolated and ligated with 3′- and 5′-ends of biotinylated, double-stranded, oligonucleotide adapters (dsODNs) with random overhangs (50 pM dsODNs to 100 ng DNA) (Table 10.1) during 24 h at 16 °C. After incubation, the ligase (Fermantas) was inactivated by heating (65 °C, 10 min) and ligation products were isolated using streptavidine sepharose beads as recommended by the manufacture (GE Healthcare). DNA was released from the beads by hydrolysis of the adapters with Sau IIIA (Fermentas) (37 °C, 1 h). After inactivation of the enzyme (65 °C, 10 min), DNA was quantified by TaqMan PCR (Bryzgunova et al. 2011).

Table 10.1 Sequences of the dsODN adaptors

	5′-sequence-3′
−3 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC **NNN**-OH
	HO-GTT TGG ATC AAA ATG GTA-OH
−2 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC **NN**-OH
	HO-GTT TGG ATC AAA ATG GTA-OH
−1 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC **N**-OH
	HO-GTT TGG ATC AAA ATG GTA-OH
0 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC TTA-OH
	HO- TAA GTT TGG ATC AAA ATG GTA-OH
+1 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC TTA-OH
	HO-**N** TAA GTT TGG ATC AAA ATG GTA-OH
+2 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC TTA-OH
	HO-**NN** TAA GTT TGG ATC AAA ATG GTA-OH
+3 5′cirDNA	biotin-TAC CAT TTT GAT CCA AAC TTA-OH
	HO-**NNN** TAA GTT TGG ATC AAA ATG GTA-OH

Results and Discussion

Whereas the DNA concentration in normal plasma samples was quite low, individual samples of cfDNA and csbDNA were pooled. It was found that the normal plasma samples mainly contained DNA fragments ~ 180 bp and fragments 8022 and 13810 bp to a much smaller extent. Mainly high molecular weight DNA was found in csbDNA from HFs (from 3765 to 14490 bp in PBS-EDTA eluate; from 1522 to 14135 bp in trypsin eluate). The length of cirDNA fragments in BCP blood was examined in individual samples. All cancer plasma samples mainly contained high molecular weight DNA fragments and fragments ~ 171 and ~ 180 bp to a much smaller extent. Short fragments were minor component of csbDNA from HF's and BCP's blood (Fig. 10.1).

To estimate the share of the short DNA in the total cfDNA, 100–1000 bp DNA were isolated from PAAG by electro elution and quantified by TaqMan PCR. Preliminary experiments demonstrate 90 % isolation recovery of PCR product for both concentrations. It was found that ~20 % of cfDNA from HFs are represented by short DNA fragments, whereas short DNA fragments composed only ~2 % in BCP blood. Short DNA fragments are rarely present in csbDNA (0.1–0.9 %) (Table 10.2).

Totally, the data obtained are close to those reported previously, although there are some inconsistencies. Short 180 bp cirDNA (Suzuki et al. 2008) as well as high molecular weight DNA fragments (Wu et al. 2002; Deligezer et al. 2003) were found in healthy donor's blood. It is considered that cirDNA of cancer or fetal origin are fragmented, but these data do not conflict with the data presented so far as we described the total cirDNA pool.

Pooled cirDNA from HFs demonstrate heterogeneity in it's termini – molecules with different 5′-protruding, 3′- protruding and blunt-ends were found (Fig. 10.2a). Pooled cfDNA from BCP blood mainly contains 3′- 3 n. and 5′- 2 n. protruding ends, whereas cancer csbDNA mainly contains 5′- 2 n. protruding ends (Fig. 10.2b) demonstrating the pressure of some overrepresented enzymes in cancer. It is known that blunt-ended breaks bearing 5′-phosphates were detected in apoptotic, but not in early necrotic cells. Over-representation of 5′-overhangs versus blunt and 3′-overhangs was shown to be typical for early necrosis (Didenko et al. 2003). It should be noted that dsODNs with random overhangs (±3 n) do not cover all DNA molecules circulating in blood. This assumption, along with non-absolute efficacy, could be responsible for incomplete additivity of the data presented.

DNA fragments are considered to be released into circulation by both normal and tumor cells mostly by necrosis and/or apoptosis. DNA termini and fragmentation analysis demonstrates that no less than 20 % of cfDNA in HFs have an

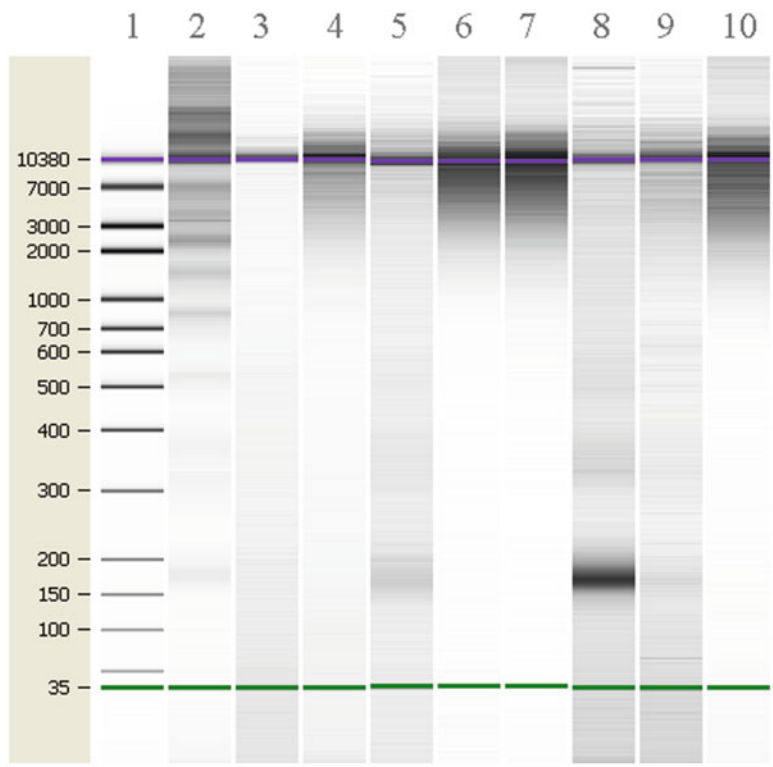

Fig. 10.1 Size distribution of cir DNA. Data of Agilent 2100 Bioanalyser ™ assay:
1. DNA ladder
2, 5. cfDNA from BCP blood
3, 6. csbDNA from TBS/EDTA eluate of BCP blood
4, 7. csbDNA from trypsin eluate of BCP blood
8. cfDNA from HF blood
9. csbDNA from TBS/EDTA eluate of HF blood
10. csbDNA from trypsin eluate of HF blood

Table 10.2 Distribution of short (100–1000 bp) and long DNA fragments (more than 1 kbp) in cirDNA from HFs and BCPs, (%)

	HFs			BCPs		
DNA fragments	Plasma	TBS/EDTA	Trypsin	Plasma	TBS/EDTA	Trypsin
100–1000 bp	18.7	0.9	0.1	2.6 ± 1.4	0.3	0.2
>1000 bp	81.3	99.1	99.9	97.5 ± 1.4	99.7	99.8

apoptotic origin. The presence of high molecular weight DNA and the abundance of 5′-overhangs in BCP circulating DNA confirm that main part of cirDNA in blood of primary cancer patients at early stage of disease (T1-2N0M0) is released from necrotic cells.

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Conflicts of Interest The authors declare no conflict of interest.

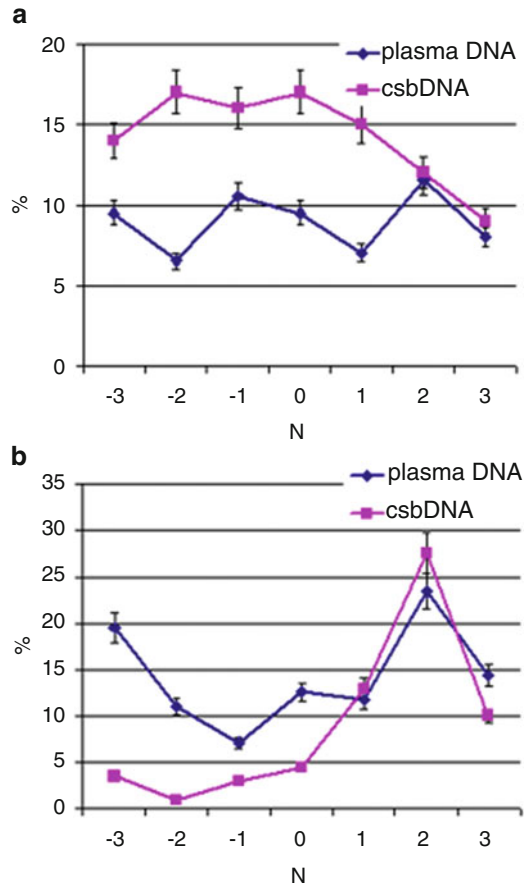

Fig. 10.2 CirDNA overhangs in blood of HFs (**a**) and BCPs (**b**) %

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Liquid Profiling of Circulating Nucleic Acids as a Novel Tool for the Management of Cancer Patients

11

Stefan Holdenrieder

Abstract

Liquid profiling is a traditional concept in laboratory diagnostics using patterns of blood-derived biochemical molecules for disease detection and characterization. Beyond protein and cellular parameters, molecular biomarkers at the DNA, RNA and miRNA level have been developed as promising diagnostic tools in metabolic and malignant diseases as new technologies for ultrasensitive profiling of nucleic acids in blood and body fluids became available. In cancer disease, they are successfully applied for the stratification of patients for individually tailored therapies, treatment monitoring and the sensitive detection of minimal residual disease. Due to its minimally invasive nature, blood-based qualitative and quantitative determinations of targeted and global molecular changes can be applied serially and complement well-established molecular tissue diagnostics. Interdisciplinary interaction between laboratory medicine, pathology and human genetics will speed up the development of liquid nucleic acid profiling as a most valuable tool for precision medicine.

Keywords

Circulating nucleic acids • Plasma • Cancer • Therapy stratification • Therapy monitoring • Resistance detection

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Circulating Nucleic Acids in Disease Detection and Management

Circulating blood is in contact with all organs in the human body and transports their genetic and metabolic products as well as molecules of cell damage or cell death. Qualitative and quantitative characterization of the biochemical constituents of the blood enables the diagnosis and staging of

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many diseases. Therefore, laboratory diagnostics in blood and other bodily fluids gained high importance in various medical disciplines. In most cases, the patterns of diverse proteins, lipids, glycans and nucleic acids were more meaningful than single markers and the understanding of the connection between the parameters became more relevant. Molecular medicine founded a new diagnostic era and has routinely been used for the identification of risk alleles in multifactorial diseases such as thrombosis or bleeding risk, of metabolic disorders as well as for pharmacogenetic testing. Molecular genetic testing efficiently complements the traditional strengths of phenotypical diagnosis and enables better understanding of complex medical issues by laboratory medicine approaches in interdisciplinary interplay with pathology and human genetics.

Beyond the identification of genetic risk factors, increasingly sensitive analytical methods have enabled the detection of circulating tumor cells (CTCs) as well as the qualitative and quantitative analysis of circulating cell-free DNA (cfDNA), RNA and microRNA in plasma and subcellular compartments like exosomes. The latter are nucleic acid-containing microvesicles actively secreted from cells with immunogenic properties. The different types and sources of circulating nucleic acids will gain increasing importance in all parts of diagnostic laboratory medicine.

During the last decades, circulating cell-free nucleic acids in plasma or serum (CNAPS) were detected in elevated concentrations after physical activity and in a variety of acute and chronic diseases such as in intensive care patients with organ dysfunctions, during sepsis, after trauma, myocardial infarction and stroke, organ transplantation and during cancer disease. Here it showed relevance for diagnosis, evaluation of prognosis and therapy monitoring of tumor diseases (reviewed in Fleischhacker and Schmidt 2007; Holdenrieder and Stieber 2009; Schwarzenbach et al. 2011; Crowley et al. 2013).

However, only disease-specific changes of CNAPS supported the better characterization and management of diseases, particularly in cancer disease. These include single mutations with loss or gain of gene sequences (e.g. in case of loss of heterozygosity (LOH) or microsatellite instability (MSI)) and numerical chromosomal aberrations; further reversible epigenetic changes in the DNA methylation pattern of promotor sequences or various histone modifications that play a major role in transcriptional regulation; finally non-coding RNA markers like the 19–24 bp microRNAs or the long-non-coding RNAs (lncRNA) and gene expression profiles (mRNA) in various blood cells and compartments (reviewed in Fleischhacker and Schmidt 2007; Schwarzenbach et al 2011; Schwarzenbach 2015; Crowley et al 2013; Gezer and Holdenrieder 2014; Holdenrieder 2014). While these new diagnostic approaches are currently still at the research level they probably will greatly enrich the possibilities in future blood-based diagnostics.

The rapid developments in the techniques of massive parallel DNA sequencing (next generation sequencing, NGS) have recently enabled in-depth characterization of cfDNA right down to single bases. Nowadays, this is mainly applied in human genetics e.g. for the characterization of fetal DNA in maternal circulation, but recently has successfully been applied in the blood-based molecular characterization of cancer disease (Chiu et al. 2011; Diaz and Bardelli 2014). In this context, exact clarification of either individual, tumor-associated or specific molecular defects were performed as well as the identification of the entire tumor signatures from plasma cfDNA and CTCs (Speicher and Pantel 2014; Lianidou 2014). Beyond pure cancer diagnosis, so-called druggable targets were detected on CNAPS that allowed the individual stratification of patients for precise pathway-specific medications (Crowley et al. 2013). Circulating tumor DNA (ctDNA) was reported to derive from necrosis, apoptosis or secretion of malignant cells or from circulating tumor cells (CTC) during minimal residual disease (Fleischhacker and Schmidt 2007; Schwarzenbach et al. 2011). It can be applied for various indications during the course of cancer disease:

(1) characterization of genetic and epigenetic changes for individual therapy stratification

(2) follow-up on molecular changes during therapy for monitoring the effectiveness of therapy
(3) detection and characterization of molecular resistance under therapy
(4) highly sensitive detection of minimum residual disease or preclinical progress.

Time- and space-dependent plasticity and complexity of tumor biology cause the necessity of genetic pattern recognition and characterization of molecular changes over time for optimal individual therapy adaptation.

Liquid Profiling – A Traditional Concept with New Implications

In the context of cfDNA and CTC analysis, the term "liquid biopsy" is frequently used for the identification of tumor characteristics (Lianidou 2010; Diaz and Bardelli 2014). However, "biopsy" is a well-established term in tissue-based diagnostics that provides important information on complex relationships of a tumor and its environment. This includes tumor classification criteria such as tumor necrosis, signs of hypoxia, tumor vascularization, phenotypic features of tumor cell heterogeneity, stroma reaction of the normal tissue and infiltration of the tumor by immune cells.

In contrast, the so-called "liquid biopsy" allows only insights in the molecular characteristics of the tumor itself by use of molecular changes of cfDNA in the blood. Similarly, tumor characteristics have been detected and monitored in the blood or bodily fluids for years by different approaches of laboratory medicine – first at the protein level as tumor markers and cellular phenotyping of leukemia cells and increasingly at the molecular level. This shows that the differentiation between tumor and normal tissue or the identification of a prognostically relevant profile of a tumor disease can be done at all levels of available biomarkers. As tumor characteristics are most efficiently assessed by multiparametric patterns, the term "liquid profiling" would express most adequately this complex diagnostic approach. Further it explains that the examination is performed in blood or bodily fluids and not in tumor tissue (Neumaier and Holdenrieder 2015) While "liquid profiling" has already widely been used in various blood-related diagnostic disciplines it now experiences a revival with respect to cfDNA and CTCs.

Companion Diagnostics for Therapy Stratification in Cancer Diseases

The molecular diagnostic characterization of cancer diseases has assumed great importance because new biological therapies target components of growth signaling pathways that are deregulated in tumor cells, e.g. due to specific mutations. Extracellularly acting antibodies or intracellularly docking small molecules block permanently expressed components and thus inhibit tumor growth. As these therapies are only effective if specific molecular changes are present, so-called driver mutations must be detected in the tumor tissue prior to therapy onset (Crowley et al. 2013; Duffy and Crown 2013). This defines which patients will most probably benefit from these targeted treatments.

Examples of the use of companion diagnostics are the detection of activating mutations (L858R or exon-19 deletion) of the epithelial growth factor receptor (EGFR) as a precondition for treatment with the EGFR tyrosine kinase inhibitors gefitinib, erlotinib and afatinib in non-small cell lung cancer (NSCLC), the detection of the activating BRAF mutation V600E for the treatment with the BRAF inhibitors vemurafenib and dabrafenib in malignant melanoma and the absence of downstream mutations KRAS and NRAS as preconditions for treatment with EGFR antibodies cetuximab and panitumumab in colorectal carcinoma (Crowley et al. 2013; Duffy and Crown 2013; Bokemeyer et al. 2015).

While the use of companion diagnostics has increased the response rates in the qualified patient groups, further improvements are necessary: In NSCLC, only 70 % of first line patients and 50 % of second line patients respond to treatment with tyrosine kinase inhibitors (TKI)

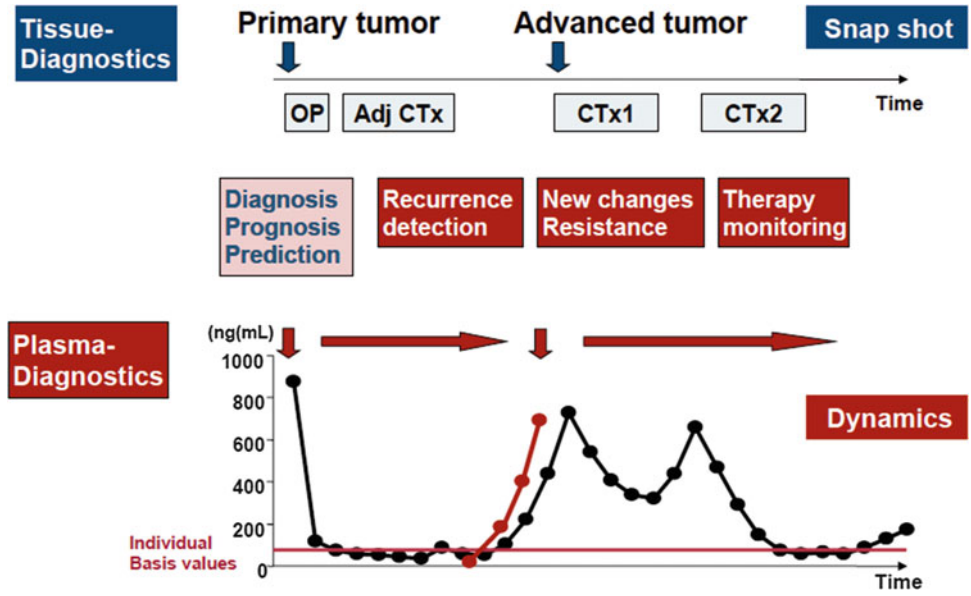

Fig. 11.1 Tissue nucleic acid diagnostics are relevant for molecular characterization of tumor disease at time of diagnosis of a primary or an advanced tumor. Identification of druggable molecular defects are preconditions for the application of individually tailored therapies. Plasma nucleic acid diagnostics support the stratification of tumor patients for specific therapies when a tissue biopsy is not available. Further serial, minimal-invasive molecular assessments are valuable for the early and sensitive recurrence detection, for the identification of new molecular changes (e.g. resistance genes) and for monitoring the response to therapies. While tissue diagnostics allow a snap shot on the molecular tumor status at a given time point, liquid profiling of circulating nucleic acids overcomes the limitations of tumor heterogeneity and plasticity and mirrors the dynamics of individual tumor biology

(Ciardiello and Tortora 2008; Petrelli et al. 2012). Also, numerous relapses and secondary progressions have been reported which, in part, result from additional resistance mutations such as the EGFR T790M mutation or the ALK-EML fusion gene – which can also be treated with crizotinib and cerotinib (Ciardiello and Tortora 2008; Crowley et al. 2013).

One reason for non-response, despite the presence of qualifying mutations, is the frequently observed molecular heterogeneity within a tumor, between primary tumor and metastases and the temporal and spatial plasticity of the tumors (Gerlinger et al. 2012).

All molecular markers from different tumor manifestations can be found in blood. Additionally, molecular changes can be followed over time with less-invasive serial blood-based diagnosis and used for monitoring therapy, early relapse diagnosis and molecular characterization of resistance (Diaz and Bardelli 2014). Lastly, liquid profiling can be performed even if the biopsy of suitable tissue material is not possible and to avoid potential biopsy-associated complications (Overman et al. 2013) Fig. 11.1.

High-Throughput Technologies in Molecular Diagnosis

Only with the development of highly parallel amplifying technologies did the ultrasensitive detection of tumor-specific nucleic acids become possible so enabling, today, a resolution of one out of 10,000 DNA molecules (Diaz and Bardelli 2014). With the commonly applied digital or clonal methods of amplification, single DNA molecules can be highly specifically amplified via emulsion PCR or cluster formation to enable selection via FACS, mass spectrometric analysis or highly parallel sequencing (Diehl et al. 2006; Chen et al. 2013; Forshew et al. 2012; Taly et al.

2013). Several current clinical studies are based on the so-called BEAMing method (Beads, Emulsion, Amplification and Magnetics), which combines digital droplet emulsion PCR with flow cytometry detection (Diehl et al. 2006, 2008). Classic next generation sequencing (NGS) approaches (e.g. Illumina, Ion Torrent), whereby single or multiple gene segments are directly sequenced, also play an important role. Here, the depth of analysis, i.e. the coverage of single gene segments, is the relevant factor for the reliable detection of single DNA molecules. Due to the, at times, small amount of DNA in blood and the small proportion of tumor DNA (sometimes less than 0.1 %) a sufficiently large initial volume of several milliliters of plasma must be collected in order to detect a tumor DNA molecule with a high degree of statistical probability. Nevertheless, a comprehensive study with 640 patients suffering from a variety of tumor diseases has shown that tumor-specific mutations of cfDNA are found in 82 % of patients with advanced tumors (excluding brain tumors) and in 55 % of patients with localized tumor stages. Of note, cfDNA was detected in plasma from the majority of tumor patients eligible for companion diagnosis (Bettegowda et al. 2014). When the frequency of KRAS mutations were compared in circulating tumor cells (CTC), cfDNA and tissues of 82 lung cancer patients, a significantly lower diagnostic sensitivity of only 52 % (at 88 % specificity) was found for CTCs vs. 96 % (at 95 % specificity) for cfDNA (Freidin et al. 2015). Bettegowda et al. also reported a significantly lower prevalence of tumor-specific rearrangements in CTCs compared to cfDNA (Bettegowda et al. 2014).

Clinical Studies on Liquid Profiling in Cancer Diseases

Liquid profiling can be effectively used in the **stratification of therapy** when a biopsy is not possible or when tissue samples cannot provide conclusive evidence. However, a high concordance of results from cfDNA and tumor tissue is postulated as a precondition for the available technologies. For the BEAMing method, such high concordance was found in several studies. Higgins et al. found, in 34 retrospective and 51 prospective cfDNA samples, a 100 % concordance with FFPE tissue samples when using BEAMing in both materials (Higgins et al. 2012). Bettegowda et al., comparing KRAS mutations in cfDNA and tumor tissue of 206 patients with metastasized colorectal cancer, found that sensitivity was 87 % with a specificity of 99 % (Bettegowda et al. 2014). Janku et al. examined 21 mutations in the BRAF, EGFR, KRAS and PIK3CA genes in patients with advanced cancer via BEAMing in comparison to standard tissue diagnosis. The obtained concordance rates were 91 % for BRAF, 99 % for EGFR, 83 % for KRAS and 91 % for PIK3CA mutations. Also, high cfDNA mutation rates were associated with poor prognosis (Janku et al. 2015). These studies show a high concordance of tumor-specific mutations in plasma cfDNA and tumor tissue. When investigating the KRAS status in the tissue and plasma of pancreatic cancer patients, we recently observed that discrepancies were also meaningful as the cfDNA was associated with a more accurate prediction of therapy response and prognosis (Holdenrieder et al. 2015). Further studies will address this phenomenon in different clinical settings.

During Tumor Therapy the volume of circulating mutant DNA correlates with the tumor mass, as shown by Diehl et al. for APC, TP53 and KRAS mutations in patients with colorectal cancer (Diehl et al. 2008). After complete surgical tumor removal, the values dropped with a half-life period of about 2 h to less than 1 % of the initial value after 24 h, whereas in the case of an existing residual tumor, they remained high. cfDNA showed a stronger dynamic and had a higher predictive value for the detection of a tumor relapse than the established tumor marker CEA (Diehl et al. 2008). Similarly convincing results were found for PIK3CA and TP53, which were detected in cfDNA of 97 % (29 of 30) of female patients with metastasizing breast cancer, while sensitivities for CTCs and CA 15–3 were found to be only 87 % and 78 %, respectively. Also, correlation with tumor load and accurate

detection of a relapse was significantly better for ctDNA (89 %) than for CTC (37 %) or CA 15–3 (50 %). In 53 % of female patients at advanced stage, ctDNA was the first marker to indicate an insufficient therapy effect (Dawson et al. 2013) with a mean lead-time of 5 months to the imaging findings. Several further studies were published on the monitoring of EGFR mutations in NSCLC patients. Here, too, a correlation between relative ctDNA changes and therapy response was found (Bai et al. 2012; Nakamura et al. 2011). These studies showed that, with known mutation status, cfDNA markers are highly suitable as "individual tumor markers" for disease monitoring during and after therapy.

The sensitive detection of detectable secondary resistance mutations has been described by Misale et al. (2012). In six of 10 patients with colorectal cancer and resistance to cetuximab and panitumumab, new KRAS mutations were detected up to 4 months prior to a CEA increase and 9 months before radiological relapse diagnosis. While the tumor cells showed a resistance to EGFR inhibitors, they remained sensitive to a combination of EGFR and MEK inhibitors, which enabled an early and individual therapy adjustment (Misale et al. 2012). Diaz et al. also reported on new KRAS mutations in nine of 24 patients treated with panitumumab, in whom originally a KRAS wild type was found and who subsequently developed therapy resistance 5–6 months after therapy onset. Based on a mathematical model, the authors were able to show that the expanding sub-clones had already been present before therapy onset (Diaz et al. 2012). Bettegowda et al. found new mutations of KRAS, NRAS and other genes of the MAP kinase signalling pathway in 23 of 24 (96 %) patients with EGFR-resistant colorectal cancer (Bettegowda et al. 2014). These findings correlate with data from tumor tissue, where up to 27 % of the non-mutated KRAS genes in exon 2 exhibited genetic variants in other segments of the gene or in *NRAS* (Schwartzberg et al. 2014; Heinemann et al. 2014). When Murtaza et al. examined genomic changes in six tumor patients using massively parallel exome sequencing, they could identify new resistance-causing and activating mutations, such as EGFR T790M, PIK3CA and RB1 (Murtaza et al. 2013).

Characterization of KRAS and EGFR resistance genes in plasma of 62 patients with metastasizing colorectal cancer with acquired anti-EGFR resistance revealed five new EGFR mutations and 27 new KRAS mutations especially in codons 61 and 146. In the respective (classified as KRAS wild type) pre-therapeutic tissue samples, these mutations were already present in low allele frequency in 35 % of the patients, correlating with poor prognosis (Morelli et al. 2015). Siravegna et al. described several acquired resistance mutations (such as KRAS, NRAS, MET, ERBB2, FLT3, EGFR and MAP2K1) in cfDNA during EGFR-inhibiting therapy. When, upon treatment, disruption of the mutated KRAS levels decreased, anti-EGFR therapy could be renewed (Siravegna et al. 2015). In a most recently published study, Garcia-Murillas et al. demonstrated in 55 female patients with localized breast cancer after successful neoadjuvant chemotherapy and curative resection, that one-off as well as serial ctDNA determination can predict occurrence of relapse – with a median time of 7.9 months before the clinically detected manifestation. Using targeted sequencing, the authors identified several new mutations in plasma ctDNA at the time of minimum residual disease with a stronger correlation with the genetic status of the detected relapse than with the genetic changes in the primary tumor (Garcia-Murillas et al. 2015). These studies show that new resistance gene cfDNA can be detected and characterized with high sensitivity. However, future clinical application of this knowledge will require the development of respective targeted therapies.

Summary

The new technical possibilities of molecular diagnostics allow sensitive and specific characterization of targeted information on circulating nucleic acids in peripheral blood that is relevant for the detection and profiling of various dis-

eases, especially malignant diseases. Apart from pure diagnosis, this approach enables the stratification of patients for individually tailored therapies, treatment monitoring and sensitive detection of minimal residual disease. As recent developments show, blood-based CNAPS diagnostics will quickly become a highly useful complement to well-established molecular tissue diagnostics as it is less invasive and overcomes limitations like tumor heterogeneity and plasticity. Interdisciplinary dialogue and interaction between laboratory medicine, pathology and human genetics will be paramount to establish CNAPS diagnostics as a most valuable tool for precision medicine in which the whole is more than the sum of its items.

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Part II

CNAPS in Foetal Medicine

12 Characterization of Human Pregnancy Specific Glycoprotein (PSG) Gene Copy Number Variations in Pre-eclampsia Patients

Chia Lin Chang, Chia Yu Chang, Da Xian Lee, and Po Jen Cheng

Abstract

Pre-eclampsia is a pregnancy-specific hypertensive disorder that affects 2–8 % of pregnancies. This disorder can lead to seizure, multi-organ failure and maternal death. The best approach to prevent pre-eclampsia-associated adverse outcomes is to be able to prevent pre-eclampsia as early as possible. Unfortunately, current diagnostic methods are ineffective at predicting the risk of pre-eclampsia during early pregnancy. In humans, low levels of a group of placenta-derived Pregnancy Specific Glycoproteins (PSGs) have been associated with intrauterine growth retardation and pre-eclampsia and there is a significant enrichment of cases with deletions in the PSG gene locus in pre-eclampsia patients. Based on these observations, we hypothesize that genomic variations at human PSG locus of maternal and/or fetal genomes may confer increased risks of pre-eclampsia. To test this hypothesis, we have recruited 90 normal control and 30 pre-eclamptic women for the analysis of fetal PSG copy number variations (CNVs).

The identification of novel PSG CNV–disease relationships will provide not only a better understanding of the pathology of pre-eclampsia but also a novel opportunity to identify patients with a high risk of developing early-onset pre-eclampsia, which has a five- to tenfold higher risk of life-threatening maternal complications and fetal demise as compared to late-onset pre-eclampsia patients.

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Keywords
Pregnancy specific glycoproteins • Pre-eclampsia • Copy number variations

Introduction

Recent discoveries have shown that a group of placenta-derived Pregnancy Specific Glycoproteins (PSGs) are essential for normal placentation and serve as autocrine/paracrine hormones to regulate trophoblast cell invasion and vascular remodeling at the feto-maternal interface. Although the role of PSGs in human pregnancy remains to be fully vetted, recent studies have shown that the deletion of genomic fragments that encompass the human PSG11 gene in the maternal genome is associated with pre-eclampsia. In addition, our recent study has shown that the human PSG gene locus (which encompasses eleven PSG genes, *PSG1-PSG11*) is enriched with large common CNVs the human PSG gene inventory ranging from 12 to 30 copies among individuals (Chang et al 2013), indicating that genomic variations at the PSG gene locus of maternal and/or fetal genomes may confer increased risks of pre-eclampsia. Taking advantage of recent progresses in massively parallel sequencing of circulating cell-free fetus DNA (cffDNA) for the diagnosis of monogenic disease in the fetus, we started to investigate genomic variations at the PSG gene locus in maternal and fetal genomes using circulating cell-free DNA in maternal plasma of normal and pre-eclamptic pregnant women during the first trimester. To sequence the highly polymorphic 550-kb PSG gene locus, we adopted a targeted sequencing strategy using an Ilumina Miseq personal sequencer.

Materials and Methods

Collection of Trophoblast Villi, Genomic DNA, and cffDNA

First-trimester villi were obtained from women undergoing voluntary elective termination or ectopic pregnancy. Blood samples were obtained from women with normal or pre-eclamptic pregnancy. Pregnancy hypertension is defined as having *de novo* hypertension (systolic blood pressure $\geq$ 140 mmHg or diastolic blood pressure $\geq$ 90 mmHg on two or more occasions at least 6 h apart after the 20th week of gestation). Pre-eclampsia is having *de novo* hypertension and proteinuria (urine protein concentration $\geq$ 300 mgL^{-1}), or substantial proteinuria ($\geq$300 mg in 24 h) at or after 20 weeks of gestation. The study was performed with approval of the IRB of Chang Gung Memorial Hospital. Volunteer donors are healthy women without either gestational diabetes or other endocrine diseases including hypothyroidism, hyperthyroidism, congenital adrenal hyperplasia, Cushing's syndrome, and hyperprolactinemia.

To investigate how PSG proteins regulate normal placentation and whether or not common PSG CNVs are associated with abnormal pregnancy, we studied (1) the effects of a PSG1 protein on trophoblast cell migration and cytokine secretion and (2) common PSG SNPs and CNVs in the maternal and fetal genomes.

Trophoblasts were isolated using a trypsin-DNase dispersion method followed by a Percoll gradient centrifugation step. To investigate the effects of PSGs on trophoblast invasion, trophoblast cell migration was analyzed with a recombinant PSG1 protein in Transwell migration chambers. Cell migration was measured 5 h after treatment. To study the effects on cytokine secretion, secreted cytokines in the media of cultured trophoblast cells were analyzed using R&D Systems cytokine arrays. For the analysis of PSG SNPs and CNVs, maternal blood and placental tissues were obtained from women with normal pregnancy or with pre-eclampsia at delivery. Fetal and maternal genomic DNAs were extracted from placental tissues and peripheral maternal blood samples, respectively. To isolate cffDNA, blood samples were drawn from candidates into K3EDTA

cell-free DNA BCT tubes. Prior to the separation of plasma, blood samples were centrifuged at 1600 g for 10 min at 4C. Plasma portion was re-centrifuged at 16,000 g for 10 min at 4 °C. DNA from plasma (≈4 ml) and buffy coat was extracted following the blood and body fluid protocol of the QIAamp DSP DNA Blood Mini Kit (Qiagen).

Results

Treatment of Cultured Human Primary Trophoblast Cells with PSG1 Led to Significant Changes in Trophoblast Cell Migration and Cytokine Secretion

Studies of trophoblast cell mobility in migration chambers showed that PSG1 significantly increases the migratory activity of cultured trophoblasts. On the other hand, analysis of levels of a variety of cytokines showed that PSG1 significantly alters the levels of MIF, RANTES, TNF-α, IL1-β, IP-10, and IL16 in trophoblast cultures.

Analysis of the PSG Gene Inventory in the Maternal and Fetal Genomes of Patients with Normal or Pre-eclamptic Pregnancy Using Next Generation Sequencing

The analysis of cffDNA in maternal plasma has been shown to be powerful approach to noninvasively diagnose monogenic diseases and aneuploidies in the fetus. Therefore, we hypothesized that the analysis of fetal PSG gene inventory in the fetus and mother could have a great potential for predicting the risk of early-onset pre-eclampsia noninvasively.

So far, we have recruited 90 normal control women and 30 pre-eclamptic women for the analysis of maternal and fetal PSG CNVs with cellular DNA or cffDNA. To sequence the highly polymorphic 550-kb PSG gene locus, we use a targeted sequencing strategy in which dozens of overlapping >10-kb genomic fragments were first amplified with high-fidelity long-range PCR followed by next generation sequencing with an Ilumina Miseq personal sequencer.

Discussion

PSG1 protein is a potent regulator of cell migration and cytokine secretion. The trophoblast cell-derived PSGs may regulate invading immune cells by modulating cytokine production in trophoblast cells, thereby helping prevent semiallotypic fetus from attacks by the maternal immune system.

The identification of potential PSG CNV–disease relationships will provide a better understanding of the pathology of pre-eclampsia and a novel opportunity to identify patients with a high risk of developing early-onset pre-eclampsia.

Acknowledgements We thank the support of a Chang Gung Memorial Hospital award (CMRPG391151-3, CLC) and the National Science Council (NSC 102-2314-B-182A-086, CLC).

Conflict of Interest None.

Reference

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13 Non-invasive Prenatal Diagnosis of Feto-Maternal Platelet Incompatibility by Cold High Resolution Melting Analysis

Marta Ferro, Hada C. Macher, Pilar Noguerol, Pilar Jimenez-Arriscado, Patrocinio Molinero, Juan M. Guerrero, and Amalia Rubio

Abstract

Fetal and Neonatal alloimmune thrombocytopenia (FNAIT) is a condition which could occur when pregnant women develop an alloimmunization against paternally inherited antigens of the fetal platelets. Approximately 80 % of FNAIT cases are caused by anti-*HPA*-1a, about 15 % by anti-*HPA*-5b and 5 % by other *HPA* antibodies. Only 2 % of the total population is HPA-1a negative (HPA-1b1b). The HPA-1a allele differs by one single nucleotide from HPA-1b allele, yet it represents around 27 % of total severe thrombocytopenias.

HPA-1 was studied in serum cDNA from 12 volunteer pregnant women to determine their *HPA*-1 genotype by HRM (high resolution melting) PCR. When an homozygous *HPA*-1 gene was detected in a mother, a COLD HRM was performed to determine whether or not the fetal genotype differs from the mother's.

The differences in the melting curve shapes allow us to accurately distinguish the three pregnants genotypes. The fetal heterozygous genotype of homozygous pregnant women was correctly detected by COLD PCR HRM in maternal serum.

HPA-1 genotyping by HRM may be a useful aproach for genotyping all pregnant women in inexpensively. Moreover, when *HPA*-1 homozygosis was detected in a pregnant woman, fetal heterozygosis may be diagnosed by COLD HRM to select pregnancies for preventive monitoring.

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Keywords
Alloimmune thrombocytopenia • Fetal circulating DNA

Introduction

Fetal and Neonatal alloimmune thrombocytopenia (FNAIT) is a condition which could occur when pregnant women develop an alloimmunization against paternally inherited antigens of the fetal platelets. There is a variety of human platelet antigens (HPA). HPA-1 is the most common platelet surface antigen implicated in caucasians. However, FNAIT occurs in approximately 1 in 1000 pregnancies. There are 34 HPAs in 6 platelet surface glycoproteins. All HPAs can be found in two different forms: a high frequency form HPA-1a and a low frequency form HPA-1b. Approximately 80 % of FNAIT cases are caused by anti-HPA-1a, about 15 % by anti-HPA-5b and 5 % by other HPA antibodies. Only 2 % of the total population is HPA-1a negative (*HPA*-1b1b). The *HPA*-1a allele differs by one single nucleotide from *HPA*-1b allele (196 T>C).

Considering the population of *HPA*-1b1b pregnant women, it is estimated that 10 % of them develop antibodies against fetal HPA-1a surface glicoprotein while, out of this 10 %, only 30 % develop fetal hemorrhagic events (severe in half of them). However, it represents about 27 % of total severe neonatal thrombocytopenias (Curtis 2015).

The objetive of this work was to establish a cost-effective way of screening to detect both *HPA*-1 homozygous genotypes in pregnant women and the fetal *HPA*-1 genotype by NIPD (non invasive prenatal diagnosis) when the pregnant women carry *HPA*-1 homozygous genoptype using COLD HRM. This is especially relevant when the mother has the most reactive form, *HPA* 1b1b.

Patients and Methods

HPA-1 was studied in serum cDNA from 12 volunteer pregnant women to determine their *HPA*-1 genotype by HRM (high resolution melting) PCR. When an homozygous HPA-1 gene was detected in a mother, a COLD HRM was performed to determine whether or not the fetal genotype differs from that of the mother.

DNA was automatically extracted from 400 µL of serum using the MagNa Pure Compact instrument (Roche Diagnostics, Basel, Switzerland) with the Magna Pure Compact Nucleic Acid Isolation Kit I according to the Total NA Plasma 100 400 V3 1 extraction protocol.

DNA amplification, HRM and COLD-HRM were performed in the same run in a Light Cycler 480 (Roche Applied Science).

Results

The differences in the melting curve shapes allow us to distinguish correctly the three pregnant women genotypes (Fig. 13.1). Figure 13.1 shows the difference plot analysis of HRM where pregnant women are classified according to their three genotypes either *HPA* 1a1a or *HPA* 1b1b in homozygosis, and *HPA* 1a1b heterozygous.

In order to detect circulating DNA (cirDNA) from a heterozygous fetus in the serum of an homozygous pregnant woman, a COLD HRM PCR was performed. In this assay the controls of every genotype analyzed were non-pregnant volunteers.

Therfore, COLD PCR HRM the increased minority genotype and fetal heterozygous

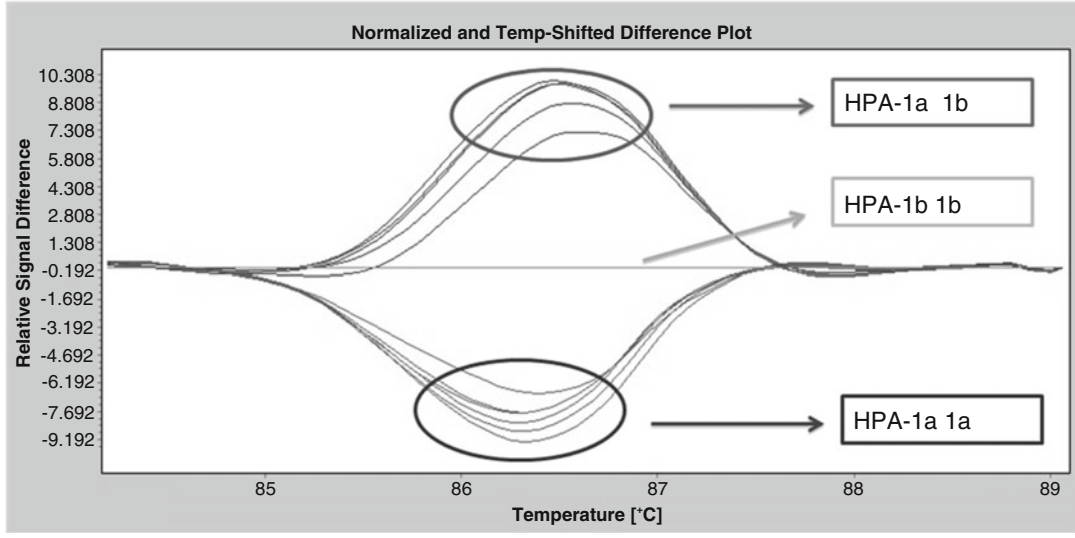

Fig. 13.1 Difference plot analysis of HRM. Pregnant women are classified according to their three genotypes HPA 1a1a, HPA 1b1b and HPA 1a1b

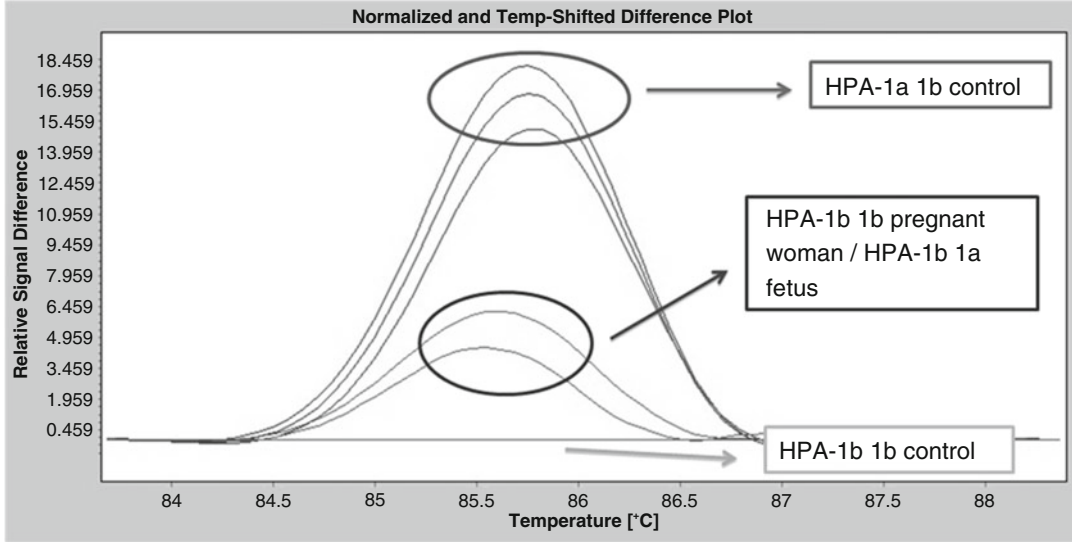

Fig. 13.2 The fetal heterozygous genotype of two pregnant women HPA1b1b was detected by COLD PCR HRM

HPA 1a gene was able to be detected. Figure 13.2 shows the detection by COLD PCR HRM of cDNA from fetal heterozygous genotype when the pregnant woman was HPA1b1b. The fetal cirDNA plot with HPA1a1b genotype clearly differs from that of HPA1b1b control women. Figure 13.3 shows the same situation when the pregnant women were HPA1a1a.

The results of one COLD PCR HRM were also analyzed by sequencing to confirm the correct genotypes.

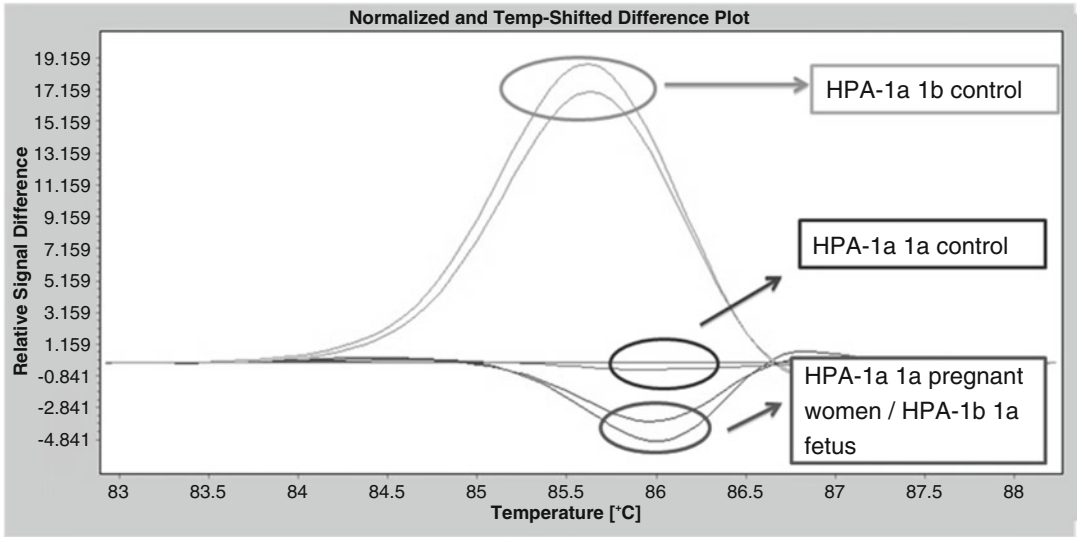

Fig. 13.3 The fetal heterozygous genotype of two pregnant women HPA1a1a was detected by COLD PCR HRM

Discussion

Fetal alloimmune thrombocytopenia may occur very early during pregnancy and fetal intracranial hemorrhage has been documented before 20 weeks of gestation, leading to death or neurologic sequelae. The prerequisite for antenatal management of FNAIT is identifying fetuses at risk when the pregnant woman is HPA1b1b. Currently, routine screening of pregnant women for HPA is not performed and there is debate regarding the need for HPA screening programmes (Curtis 2015).

HPA-1 is the principal antigen implicated in severe fetal haemorrages when alloimmunization exists and we were able to distinguish the three possible HPA-1 genotypes in pregnants women by HRM PCR. This approach is a cost-effective technique useful for a wide screening of pregnant women.

On the other hand, it is estimated that only 2 % of pregnant women have the most reactive genotype *HPA1b1b*. Our results show that HRM COLD PCR was able to detect the fetal heterozygous gene when the pregnant woman is homozygous. These data are in agreement with previous results from our group with other hereditary pathologies (Macher et al. 2012).

Other genotyping methods based on HRM analysis supporting our results have been reported (Le Toriellec et al. 2013). We propose this approach that combines in an inexpensive way to genotype all pregnant women for the detection of *HPA*-1 in homozygosis and an accurate detection of *HPA-1ab* cirDNA of the fetus in a *HPA-1aa* or *HPA-1bb* mother. Thus, if a homozygotic pregnant woman is detected, a COLD HRM PCR will be performed to determine whether the fetal *HPA*-1 genotype is heterozygous or not. This information will allow the obstetricians or gynecologists an early care of the risk pregnancies.

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14

Implementing Non-Invasive Prenatal Diagnosis (NIPD) in a National Health Service Laboratory; From Dominant to Recessive Disorders

Suzanne Drury, Sarah Mason, Fiona McKay, Kitty Lo, Christopher Boustred, Lucy Jenkins, and Lyn S. Chitty

Abstract

Our UK National Health Service regional genetics laboratory offers NIPD for autosomal dominant and *de novo* conditions (achondroplasia, thanatophoric dysplasia, Apert syndrome), paternal mutation exclusion for cystic fibrosis and a range of bespoke tests. NIPD avoids the risks associated with invasive testing, making prenatal diagnosis more accessible to families at high genetic risk. However, the challenge remains in offering definitive diagnosis for autosomal recessive diseases, which is complicated by the predominance of the maternal mutant allele in the cell-free DNA sample and thus requires a variety of different approaches. Validation and diagnostic implementation for NIPD of congenital adrenal hyperplasia (CAH) is further complicated by presence of a pseudogene that requires a different approach. We have used an assay targeting approximately 6700 heterozygous SNPs around the CAH gene (*CYP21A2*) to construct the high-risk parental haplotypes and tested this approach in five cases, showing that inheritance of the parental alleles can be correctly identified using NIPD. We are evaluating various measures of the fetal fraction to help determine inheritance of parental mutations. We are currently exploring the utility of an NIPD multi-disorder panel for autosomal recessive disease, to make testing more widely applicable to families with a variety of serious genetic conditions.

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Keywords
Non-invasive prenatal diagnosis (NIPD) • Fetal • Monogenic disorders • Cell free DNA

Introduction

Since the discovery of cell-free fetal DNA (cffDNA) circulating in maternal plasma by Lo et al. (1997), considerable progress has been made in the field of non-invasive prenatal testing and diagnosis. Conventional prenatal diagnosis requires an invasive procedure (chorionic villus sampling or amniocentesis) which has a small risk of miscarriage (Tabor and Alfirevic 2010). The presence of cffDNA has enabled safer, earlier testing to be implemented using a maternal blood sample. Clinical diagnostic tests have now been implemented for fetal sex determination (Hill et al. 2012), RHD blood group typing (Banch Clausen et al. 2014), achondroplasia and thanataphoric dysplasia (Chitty et al. 2015) and paternal mutation exclusion of cystic fibrosis (Hill et al. 2015). These methods all rely on the detection of alleles present in the fetus but not in the mother. Non-invasive definitive diagnosis of recessive and X-linked conditions, or dominant conditions where the mother is also affected, has remained challenging due to the high presence of maternal mutant allele in the plasma. However, recent advances in technology and a number of proof-of-principle studies have demonstrated the feasibility of NIPD for such conditions (Lo et al. 2010). One example of this is congenital adrenal hyperplasia (CAH), a condition where females are at risk of genital virilisation. Although maternal dexamethasone treatment reduces adverse effects, treatment is controversial due to limited long-term follow-up data and suspected side-effects. Fetal sex determination can be performed non-invasively to reduce the number of pregnancies subjected to invasive testing. New et al. (2014) recently described NIPD for CAH using next generation sequencing. The causative gene (*CYP21A2*) for CAH has a known pseudogene and as such mutations cannot be directly targeted in cfDNA due to the short (approximately 145 bp) nature. Here we describe how we have established an NIPD service in our public sector National Health Service accredited laboratory, initially for paternally inherited or *de novo* dominant mutant alleles and now recessive disorders.

Materials and Methods

Audit of NIPD Cases

An audit was performed of all clinical NIPD cases in our laboratory over an 8-year period until August 2015, for fetal sex determination, achondroplasia, thanataphoric dysplasia, paternal mutation exclusion for cystic fibrosis and a bespoke service for paternal mutation exclusion and *de novo* recurrence. Fetal sex determination is performed using qPCR and probes specific to the *SRY* gene and *CCR5* as a reference (Hill et al. 2011). For paternal mutation exclusion and *de novo* mutations, methods are now based on massively parallel sequencing, as described in Chitty et al. (2015) and Hill et al. (2015). Briefly, primers are designed targeting the mutation of interest, tailed with Illumina P5 and P7 sequences and include a patient specific index. Following 40 cycles of PCR, amplicons are cleaned, quantified and normalised prior to sequencing on an Illumina MiSeq (100 bp single-index read). Presence of fetal DNA is confirmed by HLA type, SRY markers in male fetuses or the presence of the mutant allele itself. The number of wild-type and mutant reads is counted using an Excel macro to show the presence or absence of the mutation in the fetus.

NIPD for Autosomal Recessive Conditions: Congenital Adrenal Hyperplasia (CAH)

A SureSelect Custom Enrichment Assay (Agilent Biosystems, USA) was designed to target approximately 6700 heterozygous single nucleotide polymorphisms (SNPs) flanking 7 Mb of *CYP21A2*. 1500 ng gDNA was prepared for enrichment following the SureSelect XT protocol as per the manufacturer's instructions. CfDNA extracted from 3x4ml of maternal plasma using the QIASymphony DSP Virus/Pathogen Kit, (Qiagen, Germany) was pooled and concentrated using the Qiagen MinElute PCR Purification Kit (Qiagen, Germany) and prepared for enrichment using the KAPA Hyper Library Prep Kit (KAPA Biosystems, USA) and 16 cycles of PCR. All libraries were enriched using the custom bait panel, quantified using Qubit HS Assay (ThermoFisher Scientific, USA0 and Bioanalyzer High Sensitivity Assay (Agilent Biosystems, USA). Libraries were sequenced on the Illumina MiSeq or NextSeq. FASTQ files were aligned with Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009) and duplicate reads removed with SamTools (Li et al. 2009). As described in Lo et al. (2010), the fetal fraction was calculated using SNPs homozygous in each parent for different alleles using the equation $f=(2p/(p+q))$, where p is the number of reads of the fetal specific allele and q that of the allele shared by the mother and fetus. Haplotype analysis was performed using paternal and either proband or invasive sample to link SNPs to the mutant allele. The cell-free DNA (cfDNA) sample was used to make a non-invasive diagnosis. The presence of SNPs on the paternal mutant allele in cfDNA indicated inheritance of the paternal mutation. To determine inheritance of the maternal allele against the high background of the mother's own cfDNA, relative haplotype dosage (RHDO) was applied, measuring allelic imbalance of SNPs heterozygous in the mother and homozygous in the father.

To test the approach, three families were selected where parental samples, DNA from invasive testing and cfDNA from the same pregnancy were available. The invasive sample was used to construct parental haplotypes. Using the cfDNA sample from the same pregnancy, the haplotype of the fetus was then inferred.

The approach was then applied to retrospective samples in our biobank of cfDNA from cases undergoing invasive testing for CAH, as is current practice. Three families with DNA from parents, affected proband and cfDNA from a subsequent at–risk pregnancy were available. DNA from parents and affected proband were used to construct haplotypes as above. CfDNA from a subsequent pregnancy was then analysed for NIPD and, where possible, confirmation of the non-invasive diagnosis was made by the outcome of invasive testing or testing at birth.

Results

Audit of NIPD Cases in the NE Thames Regional Genetics Laboratory, Great Ormond Street Hospital

Fetal sex-determination was offered on a research basis from 2007 when 89 cases were tested per year. Following approval for use by the UK Genetic Testing Network (UKGTN) in 2011, this has now risen to approximately 160 cases per year (Fig. 14.1).

In total, since offering NIPD for monogenic disease, 84 % of tests have been for skeletal dysplasias (achondroplasia and thanataphoric dysplasia); as NIPD use increases, invasive testing has reduced for these conditions (Fig. 14.1). Bespoke testing has been performed for ten different conditions and made up 7 % of all NIPD for monogenic disease to date, including four cases of Torsion dystonia, three of Frasers syndrome, two osteogenesis imperfecta and one case each of Rhabdoid tumour predisposition, tuberous sclerosis, Crouzon syndrome, Crouzon *acanthosis nigricans*, neurofibromatosis, autosomal recessive polycystic kidney disease and Zellweger syndrome. In the year 2014–2015, our laboratory was the biggest provider of prenatal diagnosis in the UK. Where a definitive diagnosis was made in a monogenic condition, 32 % were made using NIPD. If fetal sex determination was

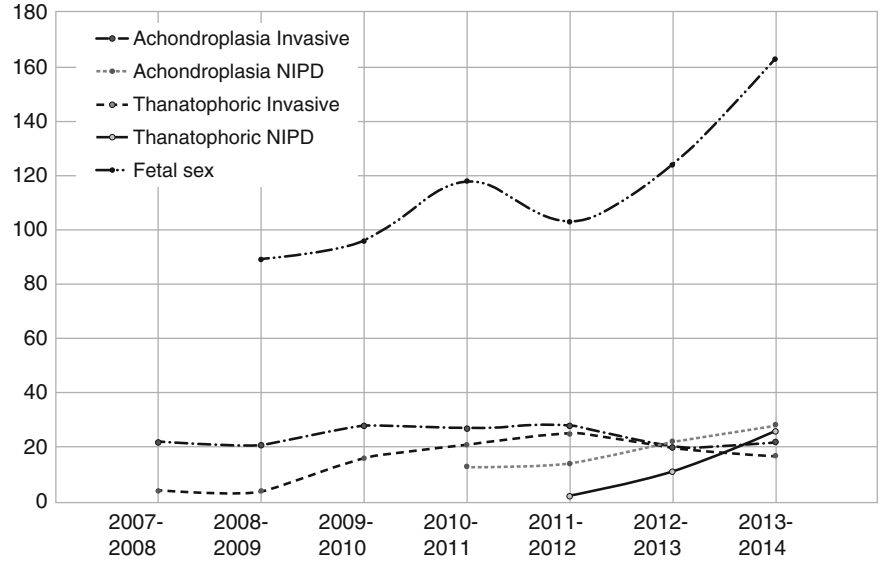

Fig. 14.1 NIPD service history for fetal sexing and skeletal dysplasia in the NE Thames Regional Genetics Laboratory

included, then 60% of our prenatal diagnostic work for monogenic disorders was done by NIPD.

NIPD for Autosomal Recessive Conditions: Congenital Adrenal Hyperplasia (CAH)

In all three normal cases, cfDNA testing correctly predicted the fetal genotype. In both CAH cases with follow-up available, the fetus was correctly predicted to be a carrier. The third CAH family declined follow-up but was predicted to be a carrier of the paternal mutation. A specific example is a couple that had an affected child due to inheritance of a maternal whole gene deletion conversion and a paternal deletion/conversion involving exons one to three. CfDNA testing using 406 informative SNPs showed a fetal fraction of 9%. A total of 947 SNPs were heterozygous in the father and homozygous in the mother. SNPs linked to the father's wild type allele were observed in the cell-free sample. RHDO using 2668 informative maternal SNPs (heterozygous in the mother and homozygous in the father) diagnosed the fetus as a carrier of the maternal mutation. Analysis indicated that a recombination event had occurred 3-prime of the mutation. DNA from the invasive sample of the same pregnancy also indicated a recombination in the same region and the diagnostic report issued on the basis of the invasive test confirmed that the fetus was a carrier of the maternal mutation.

Discussion

The increased availability of NIPD has been largely facilitated by advances in technology, namely, the advent of massively parallel sequencing. Earlier assays included polymerase chain reaction restriction enzyme digest (PCR-RED) and digital PCR (dPCR) (Chitty et al. 2011, 2013) (Lench et al. 2013). NGS has enabled NIPD service expansion to a number of conditions, including definitive diagnosis for recessive conditions. In order to do this, the fetal fraction must be calculated. A number of methods have been described including Y-chromosome markers, size differential between maternal and fetal cfDNA and heterozygous SNPs. Using heterozygous SNPs, we were able to calculate the fetal fraction and perform NIPD for the recessive condition CAH. This approach will not be necessary or feasible for all types of recessive condi-

tions. For example, for sickle cell anaemia and ß-thalassaemia, parents are frequently referred for invasive testing as a result of carrier screening in pregnancy rather than as a result of a previously affected child. In these circumstances, the haplotyping approach using a proband cannot be used. We are developing a scalable next generation sequencing assay in terms of both sample number and mutation type that can be used for the fetal fraction and diagnosis.

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Conflict of Interest None to declare.

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Part III

Biology of CNAPS

Comparative Analysis of Harmful Physical Factors Effect on the Cell Genome

15

Irina N. Vasilyeva, Valery N. Zinkin, and Vladimir G. Bespalov

Abstract

Exposure to either to low-frequency noise or ionizing radiation causes an increase in the number of chromosomal aberrations in the bone marrow cells and the level of low-molecular-weight DNA in the blood plasma of experimental animals. The dynamics of the content of low-molecular-weight DNA increasing after exposure to low-frequency noise and ionizing radiation differs significantly. Both exposures are able to provide a direct damaging effect on DNA.

Keywords

Low-molecular-weight DNA • Chromosomal aberrations • Ionizing radiation • Low-frequency noise

Introduction

The effect of radiation on the genome of the cell is well known and results mainly in the death of cells with a significant number of double breaks exceeding the reparative capabilities of the cell through a p-53 mechanism of dependent apoptosis. When repair systems are able to rectify DNA damage, p53 modulates apoptosis to protect from the appearance of mutant cells (Mathieu et al. 1999). However, there are very few reports of similar mechanisms of the effect of high-intensity noise (Silva et al. 2002; Zhou et al. 2011).

It should not be overlooked that the nature of the biological effect of noise depends not only on the level and the duration of the noise, but also on its spectrum. Industrial noises are characterized by infrasound and low frequency in their spectrum (Zinkin et al. 2013). It is shown in a number of investigations that low-frequency noise (LFN) (<400 Hz) has a certain adverse effect at the cellular, structural and organ levels. The respiratory system is the most crucial organ tending to be affected by low-frequency acoustic vibrations (Antunes et al. 2013; Zinkin et al. 2011, 2013).

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The assessment of chromosomal aberrations and dicentrics in their composition is a key component of biodosimetry in affected populations. The DNA released from apoptotic cells is specifically cleaved between nucleosomes into fragments with sizes of 180 base pairs (bp) and shows a distinct laddering pattern in electrophoretic gels (Choi et al. 2004). Such fragments are evaluated as a low-molecular-weight DNA (lmwDNA), its molecular size is 160–180 bp (Vasilyeva et al. 2011, 2015).

To compare the effect of ionizing radiation (IR) and LFN on the integrity of genome, the following evaluation criteria were used namely, the frequency of chromosomal aberrations in bone marrow cells and the indicator of cell death – the content of lmwDNA in the blood plasma.

Material and Methods

Animals: adult Wister male rats weighing 170 ± 35 g were obtained from the animal breeding farm Rappolovo (Leningrad region) and were kept under standard conditions of vivarium, in accordance with the national standard of the Russian Federation GOST R 53434–2009 "Principles of Good Laboratory Practice. All animals were kept in standard polypropylene cages, six rats in each cage, under a 14/10 h light/dark regimen at 21–23 °C. The received standard pellet laboratory diet PK-120 (Laboratorkorm, Moscow, Russia) supplemented by water *ad libitum*. The design of these experimente was approved by the ethics Committee of N.N. Petrov Research Institute of Oncology (St.-Petersburg, Russia).

Gamma irradiation involved a single treatment for 1.05 min by whole body irradiation by the unit IGUR-1 ^{137}Cs (Russia) at a dose rate of 1.9 Gy/min and a total dose of 2 Gy. Exposure to low-frequency noise: single (17 min) and multiple (17 min daily for 5 days a week during 13 weeks) treatments by the electrodynamics emitter JBL 2225 (USA), capable of generating the maximum range below 250 Hz with the sound pressure levels (SPL) of 120 dB and 150 dB.

Chromosomal aberrations: these were assessed 24 h after treatment. All animals were injected intraperitoneally with 0.2 ml of 0.025 % colchicine 2 h before cervical dislocation. Bone marrow preparations were obtained after flushing the tibia with Media 199 at 37 °C. Collected cells were centrifuged at 150 g for 6 min followed by suspending in 0.56 % KCL solution and fixation in 1:3 mix of ice-cold acetic acid and methanol. Preparations were stained for 40 min and analyzed microscopically. At least one hundred well-spread metaphases per animal were analyzed in a blind fashion.

Blood samples were collected after decapitation of animal. For each sample, plasma was separated after centrifuging at 900 g for 10 min at 4 °C. To remove remaining cells and cell debris, the plasma was further centrifuged twice at 2200 g for 10 min each. From the processed plasma samples, cell-free nucleic acids were extracted using the phenol/chlorophorm deproteininzation technique and precipitated with ethanol. The dried pellets were dissolved in deionized water (1 μl per 1 ml of initial plasma sample). Dissolved DNA preparations were incubated with RNAse and evaluated in a gradient 2/16 (%) polyacrylamide gels stained with ethidium bromide. For each sample, lmwDNA (160–180 bp) were quantified against a standard prepared using BspR1 digestion of pBR322 plasmid.

Statistical procedures. The differences in total number of chromosomal aberrations, abnormal metaphases and the mitotic index between treatments and controls were analyzed by analysis of variance, with the calculation of the F statistic and its respective p value. The significance of the discrepancies in lmwDNA was assessed using Student's criterion.

Results and Discussion

It was found that the frequency of chromosomal aberrations following exposure to either LFN for 17 min or after irradiation at the dose of 2 Gy was increased more than 10 times as compared to the control (Table 15.1).

Table 15.1 The frequency of chromosome aberrations in metaphases of bone marrow cells after a single exposure to radiation or low-frequency noise

Group	Dose	N of rats	Chromosome aberrations, %	Types of chromosome aberrations, %		
				Single	Paired	Dicentrics
				Fragments		
Control		18	0.9 ± 0.3[a]	0.3	0.6	0.0
Ionazing radiation	2 Gy	6	10.0 ± 1.4	3.2	4.7	2.1
Low-frequency noise	120 dB	6	11.3 ± 1.3	1.6	7.5	2.2
	150 dB	6	10.0 ± 1.3	1.4	7.1	1.5

[a]Statistical difference from control group $p < 0.05$

Table 15.2 The content of low-molecular-weight DNA (ng/mL) in blood plasma of rats exposed to low-frequency noise (n = 8)

Treatment	Time after the treatment	Control group	Experimental groups and SPL (dB)	
			120	150
Single	1 day	11.0 ± 5.4	84.7 ± 30.9[a]	83.5 ± 23.7[a] $p > 0.05$
	7 days	18.8 ± 1.6	90.3 ± 18.8[a]	40.4 ± 6.3[a] $p < 0.05$
Multiple	1 day	17.7 ± 1.7	644.6 ± 89.2[a]	395.6 ± 99.9[a] $p < 0.05$

[a]statistical difference from control group $p < 0.05$; $p < 0.05$ – statistical significant difference between groups

The spectrum of the studied aberrations varies. Dicentrics were practically absent from the control, but their amount was 1.5–2.2 % after the treatments. The differences in the spectrum of chromosomal aberrations after an irradiation of 2 Gy or the actions of LNF were reduced to a somewhat smaller ratio of single/paired fragments (1.6–2.1/7.5–7.1) following the action of LNF compared with irradiation of 2 Gy (3.2/4.7) (Table 15.1). Thus, the effect of LFN may result in the occurrence of DNA double strand breaks in cells.

It was specified that the effect of LNF was accompanied by an increased level of lmwDNA in the blood of the experimental animals (Table 15.2).

One day after a single exposure to LFN, the level of lmwDNA was significantly ($p < 0.05$) increased in the blood plasma of both experimental groups: 120 dB and 150 dB. Intergroup differences were not revealed in the content of this indicator according to the SPL. Seven days after the event, the level of the plasma lmwDNA remained at the same significantly higher level of the group of animals exposed to LNF with SPL of 120 dB. After this period, it began to decline, but remained above the control values in the second group of rats (SPL 150 dB). Multiple exposures to LNF resulted in an even greater increase in the level of this indicator in the blood plasma of both groups of rats. The lmwDNA content of the blood plasma increased significantly in the group of animals which were exposed to LNF with SPL of 120 dB as opposed to the group of rats with SPL of 150 dB.

Exposure to IR resulted in increases of lmwDNA content in the blood plasma with increasing doses. It should be noted that the dynamics of the increasing the content of lmwDNA after IR treatment was significantly different from that observed after the action of LFN. The concentrations of DNA in blood plasma increased with higher doses for all doses studied, from 2 to 100 Gy. But the study of plasma DNA levels in the time interval from 1 h to 3 days showed that the DNA level declined 1 day after irradiation (Vladimirov et al. 1992). The highest concentrations of lmwDNA were observed 5 h after irradiation (Vasilyeva 2001; Vasilyeva et al. 2015). At this point of time there is a linear relationship of the content of lmwDNA and exposure dose in the range from 2 to 20 Gy. Beyond the radiation dose of 20 Gy, a logarithmic relationship was registered (Table 15.3).

Table 15.3 The content of low-molecular-weight DNA in blood plasma (ng/mL) 5 h after the irradiation of animals

	Irradiation dose (Gy) in the experimental group (n = 7)					
Control group	2	4	8	20	50	100
5.5 ± 1.5	11.9 ± 5.7	25.5 ± 7.5[a]	53.2 ± 15.5[a]	138.6 ± 28.6[a]	271.6 ± 42.4[a]	338.5 ± 49.9[a]

[a]statistical difference from control group $p < 0.05$

The significant differences ($p < 0.05$) from the control was observed starting at the dose of 4 Gy. Quantification of blood plasma lmwDNA can be used as biodosimetric indicator. This indicator is informative for the radiation effects in a wide range of radiation doses from 1 to 100 Gy.

Thus, it was shown that the action of high energy LFN is accompanied by increased lmwDNA levels in rat blood plasma. It is necessary to note the following. Firstly, a single acoustic exposure causes a significant increase in the concentration of an indicator and the intensity of the reaction corresponds to a dose IR of 20 Gy. Secondly, the concentration of plasma lmwDNA continues to increase as a result of the multiple effects of LFN and exceeds almost twice the value obtained by irradiation at a dose of 100 Gy. Thirdly, the impact of LNF causes a sustained increase of this indicator in the blood plasma of rats which indicates the absence of an adaptation by the organism to this factor and, thus, its sufficiently high extremality. It is known that lmwDNA is rapidly lost from the body. Long-term preservation of its increased content indicates an ongoing cell death in rats following exposure to LNF and a substantial breach of homeostasis.

The experimental data and some knowledge of the pathogenesis of LNF action allows us to make assumptions about the mechanism underlying the appearance of lmwDNA in rat plasma. It is known that acute exposure to high intensity LNF is accompanied by structural and cellular abnormalities in the internal organs of rats, particularly in the lungs. This leads to the appearance of subpleural hemorrhage and foci of distelectasis in the lungs, hyperemia of vessels of the viscera, to an increase in mast cell and platelet degranulation as well as activation and destruction of leukocytes (Zinkin et al. 2011). Therefore, the cell death that is observed in the irregularities in the lungs described above and the direct effect on the blood cells can be regarded as the main reason leading to an increased lmwDNA in the blood plasma (Fig. 15.1).

The nature of the morphological disorders changes a little after a multiple action of LNF. The structural changes typical of a focal emphysema (interalveolar septum are thinned, airiness of the alveoli is increased, alveoli are rounded, entrances into alveoli are dilated) come to the first. The increase in blood circulation of the lungs was observed, manifested by a plethora of vascular microcirculation showing signs of separation of blood cells, marginal standing of leukocytes and clusters of red blood cells in the form "rouleaux"; there were foci of diapedesis hemorrhage. These changes in the lungs remained for a long time (Zinkin et al. 2011). They may become the structural basis for the development of such diseases as chronic nonspecific pulmonary diseases by the type of obstructive bronchitis and/or focal emphysema. Therefore, cell death, which is observed in the irregularities in the lungs described above and the changes in blood cells, can be regarded as a source of increase of lmwDNA levels in the blood (Fig. 15.2).

Conclusion

The pathological conditions involving increasing cell death and possibly apoptosis lead to an increase in the content of circulating lmwDNA (Vasilyeva et al. 2011, 2015). The increase of this indicator in the blood of various pathologies, depending on their origins, allows it to be considered as a nonspecific indicator of an increase and/

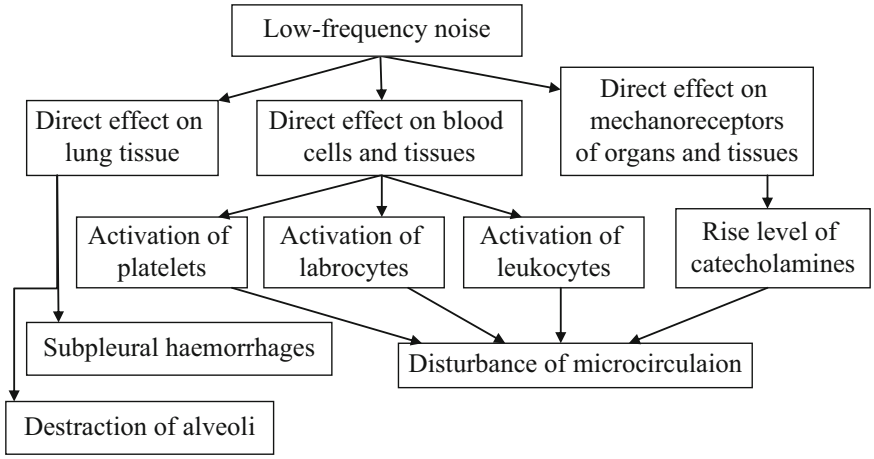

Fig. 15.1 Mechanisms of damage in the lungs during acute exposure to low-frequency noise

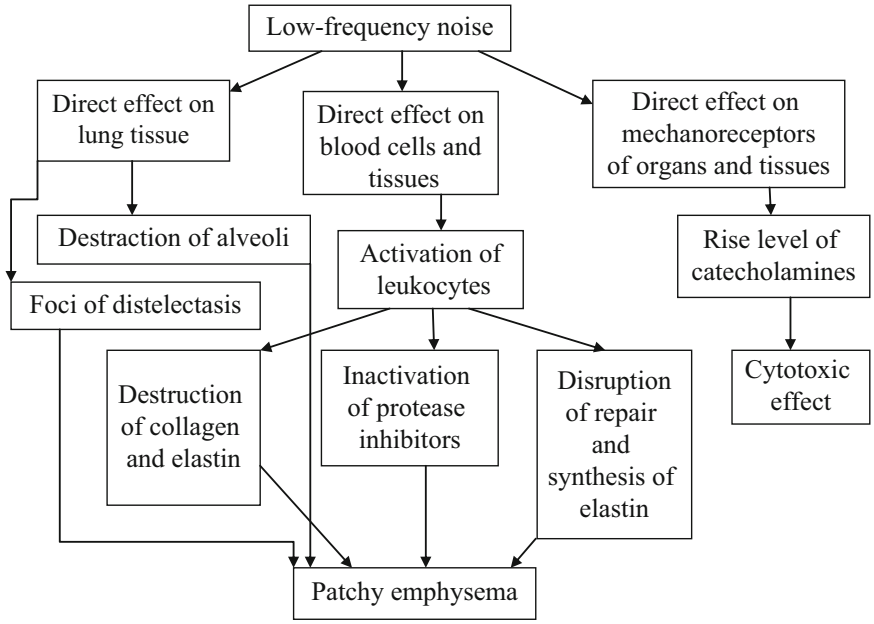

Fig. 15.2 Mechanisms of damage in the lungs during multiple exposure to low-frequency noise

or an acceleration of cell death in humans and animals.

Our data support the use of circulating lmwDNA as an informative indicator to diagnose and determine the impact of environmentally adverse physical factors (LFN and IR). LFN by analogy with IR is able to provide the direct damaging effect on DNA of cells and chromosomes; it creates certain conditions for mutagenic effects.

LFN at high levels due to the direct and indirect effect causes disturbance of microcirculation and the damage of the internal organs, especially the lungs. The pathological signs point to the

development of restrictive lung disorders of the type of focal emphysema and obstructive bronchitis.

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Heterochromatic Tandem Repeats in the Extracellular DNA

16

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Abstract

Only limited sequencing data of the normal extracellular DNA (ecDNA) are currently available. The uptake of the ecDNA by cultured cells and its integration into the host chromatin have been demonstrated. A number of membrane-bearing vesicles in plasma and serum have been shown to carry nucleic acids. The presence of Tandem Repeat (TR) in both apoptotic DNA of HUVEC culture medium and membrane-associated DNA is shown. The existence and successful application of CREST serum also show the presence of fragments of the centromeric heterochromatin together with their TR and specific proteins in blood. Apparently, pericentromeric and centromeric DNA (TR) should be part of ecDNA in all cases.

Keywords

Extracellular DNA • Tandem repeats • Centromeric proteins • Apoptosis • Membrane-associated DNA

Introduction

The fact of the extra-cellular DNA (ecDNA) circulating in bodily fluids in higher eukaryotes was known long ago, but it attracts less the attention of biologists than of physicians (Anker et al. 1975; Vasioukhin et al. 1991, 1994). Now, these studies are becoming increasingly important for the development of minimally invasive diagnostics.

Sequencing of Extracellular DNA

Genome assembly and new sequencing methods have shed light on the problem of the sequencing of ecDNA (Murtaza et al. 2013). In pioneering work, the deep sequencing of the exomes of plasma DNA was used as a platform for the

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non-invasive analysis of tumor evolution during systemic cancer treatment. Sample sequencing with high tumor burden in blood allows the assessment of clonal heterogeneity and selection. Synchronous biopsies were also analyzed, confirming the genome-wide representation of the tumor genome in plasma. Quantification of the allele fractions in plasma has allowed identification of an increased representation of mutant alleles in association with the emergence of therapy resistance. Serial analysis of cancer genomes in plasma constitutes a new paradigm for the study of clonal evolution in human cancers (Murtaza et al. 2013). Unfortunately, only limited sequencing data of the normal ecDNA are available.

Currently, ecDNA of healthy donors, pregnant women, patients with prostate cancer, the culture medium of HUVEC and irradiated rats have been sequenced (Vasilyeva et al. 2015). Among the first to be sequenced was ecDNA of patients with systemic lupus erythematosus (SLE). It was found that the ecDNA belongs mainly to the genomic DNA, is of nucleosomal structure and is enriched by GC-pairs and Alu repetitive elements. It is believed that these features are due to the properties of either the DNA in the circulation or the mechanism of apoptosis. A clinically significant increase in circulating DNA is noted not only for SLE, but also for other rheumatic diseases such as autoimmune liver disease, autoimmune vaculitis and autoimmune disorders of connective tissue.

The Uptake of the Extracellular DNA

The uptake of the ecDNA by cultured cells and its integration into host chromatin have been demonstrated (Mittra et al. 2015). Fragmented DNA and chromatin (DNAfs and Cfs) isolated from the blood of cancer patients and healthy volunteers are readily taken up by a variety of cells in culture to be localized in their nuclei within a few minutes. The intra-nuclear DNAfs and Cfs associate themselves with host cell chromosomes to evoke a cellular DNA-damage-repair-response followed by their incorporation into the host cell genomes. When isolated Cfs were characterized by electron microscopy, they were found to retain the typical beads-on-a-string appearance of nucleosomes. Fragments were fluorescently labelled and added in culture medium of mouse NIH3T3 cells. Numerous fluorescent signals of varying sizes could be detected in the cytoplasm and nuclei of the treated cells. Moreover, single-cell clones derived from cells treated with DNAfs and Cfs were established and analysed by whole genome sequencing of the transformed cells. Multiple human DNA insertions were detected. FISH with human whole genomic and human pan-centromeric probes showed that the signals were co-localize on chromosome arms and sub-telomeric regions. This indicated that DNAfs and Cfs inserted as concatamers and often harboured centromeric sequences (Mittra et al. 2015). Numerous fluorescent pan-centromeric signals imply that TRs constitute a significant portion of plasma DNA.

Excretion of Extracellular DNA

The main sources of ecDNA could be: (1) exosomes from resting cells, (2) microvesicules from activated cells; (3) apoptotic bodies (Rykova et al. 2012). A number of membrane-bearing vesicles in plasma and serum were shown to carry nucleic acids (Rykova et al. 2012). Apoptotic bodies (from 1 to 5 μm represent the condensed remnants of apoptotic cells. Micro-particles (from 200 to 1000 nm) originate by either blebbing or shedding of plasma membranes of diverse cell types in response to activation, injury and early apoptosis. Exosomes (from 30 to 90 nm) result from the release of the late intracellular endosomes. Membrane-bearing particles carrying nucleic acids apparently have multiple mechanisms of binding to the circulating blood cells. As a result of this association, nucleic acids can stay reversibly bound to the cell surface or can be incorporated by cells either in a receptor-mediated way or as a result of fusion between particle and cell membranes (Rykova et al. 2012).

Extracellular DNA from Apoptotic Cells

Apoptosis is well studied as a set of consecutive events and mechanisms resulting in DNA degradation, their circulation in the blood and excretion. Apoptosis was experimentally induced in human umbilical vein endothelial cells (HUVEC) and the pool of cell-free apoptotic DNA present in the culture medium (ecDNA) was sequenced and used for FISH (Morozkin et al. 2012). Both methods showed the asymmetry of repetitive sequence distribution in ecDNA: (1) significant enrichment of pericentromeric tandem repeats (TR), the most of which is HS3 (human satellite 3), but a decrease of centromeric alpha satellite; (2) enrichment in Alu (SINE), but a decrease of LINEs. Similar results have been reported for the serum of healthy donors (Beck et al. 2009).

Membrane-Associated DNAs

It is shown that TRs are attached to the inner side of the plasma membrane as 6 kb fragments that are identified by comparison with the whole genome shotgun assemblies (Cheng et al. 2012). These fragments may enrich ecDNA especially in apoptotic cells. A stable WIL_2 –CG cell line, which expressed a chitin-binding domain on the cell surface, was constructed from the human diploid B-lymphocyte cell line WIL_2 to perform the unique experiment. The membrane fragments were bound to chitin magnetic beads to ensure the purity of the membrane fraction and to avoid nuclear DNA contamination. Deep sequencing of the membrane-associated DNA showed the enrichment of both peri-centromeric simple satellite repeats (TR) and alpha satellites. The type of alpha satellite and its affiliation to the centromere are not defined. It is shown also that only RNA polymerase II of a special type can transcribe the membrane associated DNA and that DNA comprised of TR (Cheng et al. 2012). Thus, the long debated question of the existence of membrane associated DNA is positively solved in this work, but the enrichment of this fraction with TR is striking. Heterochromatin, which contains TR, remains the most enigmatic part of the genome. The presence of TR in the membrane associated DNA makes its function even more puzzling.

Centromeric Proteins in Blood Plasma

Three centromere proteins, CEN-A, CEN-B and CEN-C have been identified as major autoantigens in certain rheumatic diseases. Antibodies against them were found in certain patients with rheumatic diseases (Earnshaw et al. 1984). The name of the serum is CREST – a syndrome of scleroderma – calcinosis, Raynolds phenomenon, esophageal dysmotility, sclerodactyly, telangiecstasia. CREST was used to identify centromeres on the chromosomes (Fig. 16.1a, b). This serum contains three proteins, all of them are DNA binding and connected with centromeric DNA. CENP-A and CENP-B have been well investigated. The proteins were cloned and antibodies against each of them were prepared (Fig. 16.1c, d). Not only nucleosome DNA is found in the blood of patients with SLE (Van Helden 1985; Herrman et al. 1989; Winter et al. 2015), but antibodies against centromeric proteins are also found. So, fragments of the centromeric heterochromatin, together with their TR and specific proteins, are also components of ecDNA.

TR in Extracellular DNA

It would seem that pericentromeric and centromeric DNA (TR) should be the part of the ecDNA in all cases. However, the question about TR presence in ecDNA remains unsolved in most of the cases. Most authors compare ecDNA sequences with the reference genome. The human assembled genome was used for an ecDNA comparison. The large regions of classic heterochromatin are poorly covered by the assembled sequences, the chromosomes having prolonged TR arrays at the centromeric and sub-telomeric regions. This is the reason as to why these regions

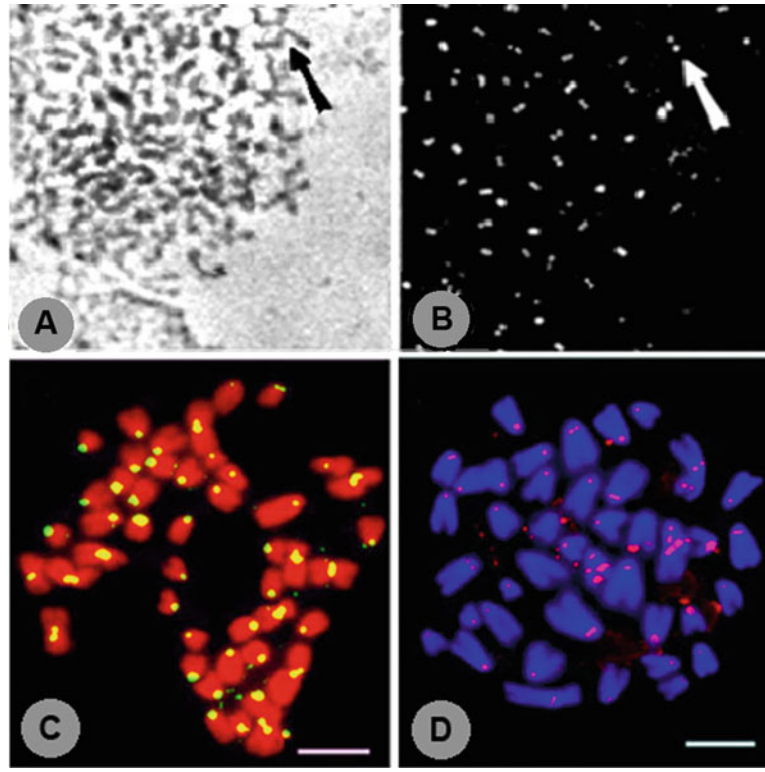

Fig. 16.1 (**a**, **b**) – CREST serum testing on HeLa metaphase plates (From Earnshaw et al. 1984). (**a**) – Phase contrast microscopy, (**b**) – CREST immunofluorescence; one of centromeres pairs is marked by an *arrow*. ×1.130. (**c**), (**d**) – mouse L929 cell line metaphase plates. (**c**) – Staining with CENP-B antibodies; (**d**) – FISH showing minor satellites. Bar – 5 μm (From Kuznetsova et al. 2007)

are difficult to assemble. In the assembled genomes, chromosomes end abruptly in 3 Mb gaps reserved for centromeric regions. Human TRs are only roughly classified. DNA attached to the inner part of the cell membrane as ~6 kb fragments represents peri-centromeric TR when compared to the raw WGS\WGA database, but not to the reference genome (Cheng et al. 2012). Transposable elements constitute no less than 48 % of the human assembled genome and their positions have been determined. Alu repeats (SINE) are located mostly to the gene-rich regions, while LINEs enrich facultative heterochromatin as evidenced by bioinformatics data (Waterston et al. 2002) and FISH (Solovei et al. 2009). Transposable element positions in both mouse and human genomes are very similar and cause the synteny together with genes (Waterston et al. 2002). On the contrary, TRs are species-specific and there is little in common between the centromeric human alphoid satellite and the mouse Minor satellites as well as between pericentromeric human big or "simple" satellites (HS3) and mouse Major satellites. These differences allow the tracing of the incorporation of human ecDNA into mouse cells (Mittra et al. 2015).

The absence of TR in genomes, and even the absence of their classification, limits the comparison. Satellite DNAs were identified about 50 years ago as an additional, "satellite" fraction of genome DNA during centrifugation on a CsCl2 gradient. Its feature is the organization into tandemly repeated monomers. The term "TR" with precise monomer length, GC content and array length make it possible to formalize the definition in order to work with sequence databases in spite of uneven "satellites" (Komissarov et al. 2011). TR DNA represents a significant portion of any eukaryotic genome (up to 10 %), and is situated in sub-telomere and centromere heterochromatin regions. Although historically referred to as "junk DNA", TRs appear to provide unique structural and functional characteristics due to their tandem organization. Centromeres from fission yeast to humans contain TRs while pericentromeric

regions enriched in TRs appear to be critically important for establishing heterochromatin formation and proper chromosome segregation. There is some evidences mentioned above for the TR presence in ecDNA though this fact could not be clarified without a proper TR classification. TR nature and functions have been poorly investigated considering their number in the genome. Given that TR happens to be one of the main components of the ecDNA, it might help to shed light on the whole of TR biology in the genome.

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Conflict of Interest No conflict of interest to declare.

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17

A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities

Janine Aucamp, Abel Jacobus Bronkhorst, and Piet J. Pretorius

Abstract

The quantitative and qualitative differences of circulating nucleic acids (cirNAs) between healthy and diseased individuals have motivated researchers to utilize these differences in the diagnosis and prognosis of various pathologies. The position maintained here is that reviewing the rather neglected early work associated with cirNAs and extracellular vesicles (EVs) is required to fully describe the nature of cirNAs. This review consists of an empirically up-to-date schematic summary of the major events that developed and integrated the concepts of heredity, genetic information and cirNAs. This reveals a clear pattern implicating cirNA as a homeostatic entity or messenger of genetic information. The schematic summary paints a picture of how cirNAs may serve as homeostatic genetic entities that promote synchrony of both adaptation and damage in tissues and organs depending on the source of the message.

Keywords

Circulating nucleic acids • Extracellular vesicles • Genetic homeostasis • Metabolic DNA • Bystander effect • Genometastasis

Introduction

Since the discovery of cirNAs in human plasma in 1948, there has been considerable amount of research regarding their diagnostic applications (Fleischhacker and Schmidt 2007). Despite the progress made, there are still inconsistencies that bolster clinical application and this is mainly due to a lack of standard operating procedures in the storage, extraction and processing of cirNAs. However, our lack of knowledge regarding the origin and purpose of cirNAs is an equal culprit. The aim of this review is to illustrate the development of cirNA research in order to elucidate cirNA as homeostatic entities or messengers of genetic information. The

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position maintained here is that reviewing the rather neglected early work associated with cirNAs and extracellular vesicles (EVs) is required to fully describe the nature of cirNAs. The review consists of an empirically up-to-date schematic summary of a few of the major events that developed and integrated the concepts of heredity, genetic information and cirNAs. This reveals a clear pattern implicating cirNA as a homeostatic entity or messenger of genetic information.

Homeostasis and DNA

DNA is vulnerable to change or damage, especially during transcription and replication. These processes are highly regulated to prevent errors from occurring, but changes can still occur and our living environment provides additional challenges to genome integrity. If all other body functions utilize homeostatic functions to maintain stability and balance, is it possible that there can be some form of homeostatic regulation for genetic information?

In 1954 Michael Lerner coined the term "genetic homeostasis", referring to the ability of a population of organisms to equilibrate its genetic composition and to resist sudden changes (Hall 2005). As the term stands, it is not really applicable to the article's aim, unless one refers to different organs, tissues and cell types as populations within the body of an organism. As with populations of organisms, genetic changes do not always occur uniformly throughout the organs and tissues of the body, but in most cases begin as isolated incidences. These changes, however, can spread to and affect nearby cells and tissues. Take the bystander effect, for example, which refers to the effect of information transfer from targeted cells exposed to damaging agents of a physical or chemical nature to adjacent cells (Ermakov et al. 2013). Targeted UV irradiation results in the release of clastogenic factors by irradiated cells that can induce apoptosis and necrosis in adjacent non-irradiated cells. These clastogenic factors have been identified as extracellular DNA (Ermakov et al. 2011) and their effects have also been found to persist in the progeny of irradiated cells that survived irradiation (Seymour and Mothersill 2000).

The Four Paradigms of the History and Development of cirNAs and EVs

The question now is whether cirNAs can serve as homeostatic entities or messengers to promote stability and equilibrium of genetic data among a population of tissues/organs. We believe this is the case and there are several instances in the discovery and development of cirNAs and extracellular vesicles (EVs) that strongly indicate this. The history and development of cirNAs and EVs consist of four main topics or paradigms, namely heredity, DNA, messengers and the cirNAs and EVs (see Fig. 17.1).

Conclusions

To conclude we ask again, can cirNAs serve as homeostatic entities or messengers to promote stability and equilibrium of genetic data among a population of tissues or organs? The answer is yes. According to our empirically up-to-date schematic summary of the history and development of cirNA research and the contributions of EV research:

(i) Darwin coined the idea of free moving updated data particles originating from all tissues;
(ii) Griffith and Avery showed us that these particles are nucleic acids and can transfer beneficial information from one place to another and can be inherited;
(iii) De Vries, Bell, Stroun, Pelc, Anker and Gahan showed us that these particles are not necessarily for heredity, but to convey messages;
(iv) Pelc showed us that metabolic DNA, and therefore spontaneously released DNA or virtosomes (if metabolic DNA serves as

Fig. 17.1 Empirically up-to-date schematic summary of a few of the major events that developed and integrated the concepts of heredity, genetic information, cirNAs and EVs. The relationships of the four paradigms of cirNA research are illustrated: Heredity (*black* with *white font*), DNA research (*white*), messengers (*dark grey* with *black font*) and cirNA and EV research (*dash lines*). Areas with other combinations of *dash lines* and *fonts* represent data that fall under more than one of the four categories

their precursors), are separate entities from our genetic DNA;

(v) The transcession studies and subsequent genometastasis and exosome research showed us that cirNA release becomes prominent when change occurs (e.g. bacterial exposure leading to transcription of bacterial DNA into recipient cells, cancer mutations, epigenetic changes, damage and/or repair due to stressors such as irradiation exposure);

(vi) CirNAs contain the changes or mutations of the parent tissue;

(vii) Garcia-Olmo's patent showed us that it is not only cirNAs related to diseases, disorders and/or damage that can induce change;

(viii) Genometastasis and the bystander effect showed us that these cirNAs can transfer change from one place to another and can become persistent in following generations. CirNas could, therefore via horizontal gene transfer, serve as homeostatic entities or messengers of genetic information.

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Conflict of Interest The authors declare no conflict of interest.

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18 Comparison of MicroRNA Content in Plasma and Urine Indicates the Existence of a Transrenal Passage of Selected MicroRNAs

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Abstract

MicroRNAs (miRNAs) in urine are examined as potential biomarkers. We examined the urine samples from 70 individuals (45 males, 25 females, mean age 65 years, range 20–84 years). Of the urine donors, 15 were healthy volunteers, 5 were patients with non-cancer diseases, 50 were patients with different stages of bladder cancer. To examine the spectrum of miRNAs in the cell-free fraction of urine, TaqMan Human miRNA Array Card A v.2.1 was used. A set of 30 miRNAs were found that are constantly present in urine supernatants independently of sex, age and health status of the subjects. We compared this set with miRNAs found in plasma, expressed in kidney and genito-urinary tract. Our results indicate that some miRNA could be transferred from the circulation into urine.

Keywords

MicroRNA • Urine supernatant • Plasma • Genito-urinary tract • Transrenal passage of microRNAs

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Introduction

MicroRNAs (miRNAs) in urine are examined as potential biomarkers (Wang et al. 2012; Erbes et al. 2015). In the field of forensic medicine, the lists of miRNAs specific for different bodily fluids were reported, but urine was, surprisingly, not analyzed intensively in this context (Silva et al. 2015). We determined the cell-free miRNA characteristics for samples of urine supernatants regardless of sex, age and health status of the examined individuals. To evaluate the potential sources of the most abundant miRNAs found in urine supernatants, we compared the list of such miRNAs not only with miRNAs reported to be highly abundant in plasma and venous blood (Silva et al. 2015), but also with lists of miRNAs expressed in plasma, kidney and genito-urinary tract according to the miRWalk database (Dweep et al. 2011).

Material and Methods

We examined the urine samples from 70 individuals (45 males, 25 females, mean age 65 years, range 20–84 years). Of the urine donors, 15 were healthy volunteers, 5 were patients with non-cancer diseases, 50 were patients with different stages of bladder cancer. All subjects provided written informed consent according to the approval of the local ethics committee. The second morning urine was analyzed in all subjects. Fifty ml of urine of each subject was collected into a tube with 1.5 ml RNA Later (Qiagen, Valencia, CA, USA) and centrifuged at 4000 rpm for 10 min at 10 °C. The supernatant was then poured into a new tube and frozen at −20 °C.

To examine the spectrum of miRNAs in the cell-free fraction of urine, TaqMan Human miRNA Array Card A v.2.1 (Applied Biosystems™, CA, USA) was used: 1 ml of urine supernatant was used for the isolation of miRNAs by the Urine microRNA Purification Kit (Norgen Biotek Corporation, Canada) according to the recommendation of the manufacturer. The purified miRNA was immediately used for reverse transcription performed according to the protocol of TaqMan® MicroRNA Reverse Transcription Kit with the Megaplex™ RT primer Human Pool A (Applied Biosystems™, CA, USA). The cDNA was pre-amplified according to the protocol of TaqMan® MicroRNA Pre-amplification with the Megaplex™ PreAmp primer Human Pool A (Applied Biosystems™, CA, USA).

The results were analysed with Expression Suite Software v1.0.3 (Applied Biosystems™, CA, USA), and qBase+© v2.4 (Biogazelle, Belgium). Three miRNAs (miR-191, miR-28-3p and miR-200b) chosen by the geNorm analysis within qBase+© programme v2.4 were used for the normalization of the expression rates. The Venn diagram was constructed using the web-based tool (http://bioinformatics.psb.ugent.be/webtools/Venn).

Results

The most abundant microRNAs that were found in all examined urine supernatants under the detection limit of the 35th Ct were: let-7c, let-7e, miR-100, miR-106a, miR-125b, miR-146a, miR-146b-5p, miR-17, miR-191, miR-192, miR-194, miR-197, miR-19b, miR-200b, miR-200c, miR-203, miR-204, miR-21, miR-24, miR-26a, miR-27b, miR-28-3p, miR-29a, miR-30b, miR-30c, miR-31, miR-532-3p, miR-886-3p, miR-886-5p, miR-99b. This list of 30 miRNAs present in urine supernatants of all the individuals examined was compared with the lists of miRNAs extracted from the miRWalk database as expressed in plasma (245 miRNAs), kidneys (150 miRNAs) and genito-urinary tract (117 miRNAs). The results of this comparison are represented in Fig. 18.1, the urine miRNAs with an unambiguous origin in genitourinary tract, plasma and kidneys and/or plasma are listed here.

We compared further the list of miRNAs found repeatedly below the detection limit of the 35th Ct in urine supernatants with the sets of miRNAs which were selected for forensic purposes for bodily fluid determination as the most

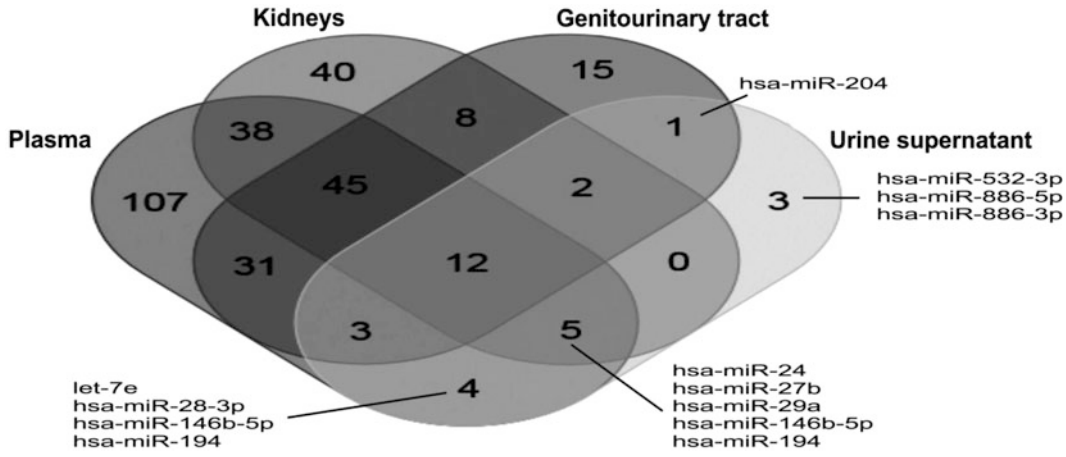

Fig. 18.1 Overlaps among sets of miRNAs expressed in plasma, kidneys, genitourinary tract and our set of miRNAs abundant in urine supernatants

Table 18.1 MiRNAs reported as abundant in plasma and venous blood (Silva et al. 2015) found in urine samples under the 35th Ct repeatedly

Abundant in plasma	No.	Abundant in plasma	No.	Abundant in blood	No.	Abundant in blood	No.
miR-135a	61	miR-508-3p	12	miR-16	69	miR-185	42
miR-139-3p	2	miR-518f	38	miR-20a	69	miR-451	58
miR-182	43	miR-519d	13	miR-106a	70		
miR-224	58	miR-551b	11	miR-126	67		
miR-373	6			miR-150	60		

No. number of urine samples in which the miRNA was detected in this study

abundant in plasma and venous blood (Silva et al. 2015). The results are summarized in Table 18.1.

Discussion

A set of 30 miRNAs were found that are constantly present in urine supernatants independently of sex, age and health status of the subjects.

We compared this set with miRNAs found in plasma, expressed in kidney and genito-urinary tract to decipher the potential origin of these molecules in urine supernatants with regard to their usefulness as specific biomarkers. We found large overlaps among compartments and only a few miRNAs with potentially detectable origin.

In urine supernatants, we detected also miRNAs reported as highly abundant in plasma and venous blood and therefore described as useful for forensic discrimination of these body fluids.

Our results indicate that some miRNA could be transferred from the circulation into urine. Further research in this area may elucidate the mechanisms of renal clearance of miRNAs.

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Conflict of Interest The authors declare no conflicts of interests.

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A Quantitative Assessment of Cell-Free DNA Utilizing Several Housekeeping Genes: Measurements from Four Different Cell Lines

19

Janine Aucamp, Abel Jacobus Bronkhorst, Johannes F. Wentzel, and Piet J. Pretorius

Abstract

Quantitative real-time PCR (qPCR) is regularly used to quantify cell-free nucleic acids (cfNAs) in order to identify biomarkers for various pathologies. However, studies have shown notable housekeeping gene expression variation between healthy and diseased tissues and treated versus untreated cell lines. The release of housekeeping genes by four cell lines was investigated and the housekeeping gene expression between cfNAs and mRNA of the cell lines was observed in order to elucidate their relationship.

Keywords

Housekeeping genes • Cell-free DNA • qPCR • Cell culture

Introduction

The targeting of housekeeping genes as biomarkers in cell-free nucleic acids (cfNAs) quantification is becoming quite common (Mouliere et al. 2015), but which gene serves as the most appropriate target? In quantitative real-time PCR (qPCR), housekeeping genes are primarily used as internal controls, but studies have shown notable expression variation between healthy and diseased tissues (Rubie et al. 2005) and treated versus untreated cell lines (Powell et al. 2014). These variations contradict the prerequisites for internal control housekeeping genes (Kozera and Rapacz 2013). In terms of pathology biomarker detection, however, expression variation is welcomed and the internal control prerequisites can be used to optimize cell-free DNA (cfDNA) quantification. This study focuses on the utilization of housekeeping gene expression analysis in the optimization of cfDNA quantification and the elucidation of the relationship between cfDNA and mRNA in cell lines.

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Materials and Methods

The total RNAs of melanoma (A375), osteosarcoma (143B), rhabdomyosarcoma (RD) and skin fibroblasts (ZANLP) were extracted with the NucleoSpin RNA II extraction kit (Machery Nagel), the concentrations and purity determined with the Nanodrop ID-1000 spectrophotometer (Thermo Scientific) and cDNA synthesised using the High Capacity RNA-to-cDNA kit (Applied Biosystems). The cfDNA of culture medium from the four cell lines was extracted with the NucleoSpin Gel, PCR Cleanup kit (Machery Nagel) and binding buffer NTB. The primers and probes of seven housekeeping genes from the GeNorm Reference Gene Selection Kit (Primerdesign) and β-globin (IDT, Whitehead Scientific) were used to evaluate the occurrence of housekeeping genes of the four cell lines and qPCR was performed with the RotorGene Q (Qiagen) according to the instructions of the GeNorm kit manual. Fifteen cfDNA replicates and eight cDNA replicates were prepared and the thresholds chosen to produce Ct values from the resulting raw fluorescence data was 0.028 for the cfDNA samples (as used in previous quantitative PCR studies) and 0.04 for the cDNA samples (Fig. 19.1).

Results and Discussion

ACTB was identified as the most stable gene in both cfDNA and mRNA and β-globin was stable in cfDNA, correlating with a recent study of

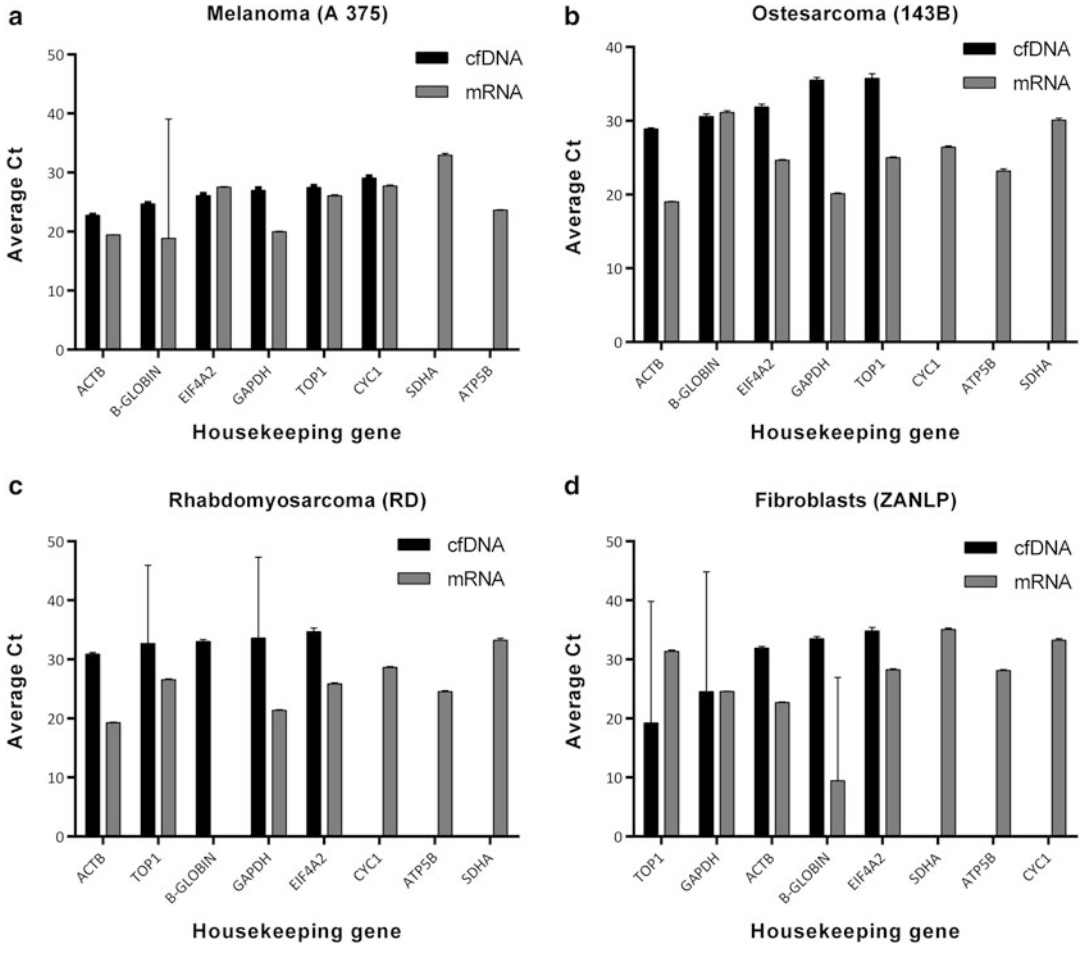

Fig. 19.1 Average Ct values of housekeeping genes that occur in the cfDNA and mRNA of the four cell lines. The cirDNA concentrations used remained constant in all four cell lines. The concentrations of cfDNA used represent the total yield of cfDNA for each cell line. Low Ct values indicate higher levels of initial PCR template and earlier cycle amplification

maternal- and fetal-derived DNA (Yang et al. 2015). The remaining housekeeping genes showed considerable variability in some cell lines and evaluating their use as controls beforehand is strongly recommended. There was little variation in the occurrence of housekeeping genes between the cfDNA of cancerous cell lines and the healthy fibroblasts. Both the cfDNA of the cancerous cell lines (with the exception of A375) and healthy fibroblasts did not release housekeeping genes related to cellular energy metabolism (SDHA, ATP5B and CYC1), but mRNA one results did show that they were expressed. The analysis of control gene stability in cfDNA samples rather than genomic DNA for qPCR will promote the optimization of cfDNA quantification.

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Conflict of Interest The authors declare no conflict of interest.

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20 Oligodeoxynucleotide Analogues of Circulating DNA Inhibit dsRNA-Induced Immune Response at the Early Stages of Signal Transduction Cascade in a Cell Type-Dependent Manner

Anna V. Cherepanova, Zhanna K. Nazarkina, and Pavel P. Laktionov

Abstract

Oligodeoxynucleotide (ODN) analogues of cell-surface-bound circulating DNA inhibit the dsRNA-induced production of pro-inflammatory interleukin 6, interferon beta and antibacterial peptide beta-defensin 2 not only in human gingival fibroblasts, but also in human primary endothelial and transformed cells (Hela and A431). ODN analogues do not effect dendritic cells activation by poly(I:C). The data obtained indicate that the early stages of the signal transduction cascade are violated by ODN analogues and the effects depend on the cell type.

Keywords

Circulating DNA • Double-stranded RNA • Interleukin • Interferon • Innate immunity

Introduction

DNA from different sources including free and cell-surface-bound circulating DNA inhibit the poly(I:C)-activated production of pro-inflammatory interleukins (IL-6 and IL-8) in human primary gingival fibroblasts, the inhibiting efficacy depending on the DNA sequence (Cherepanova et al. 2013). To identify the mechanisms of the immuno-inhibiting action of DNA, we investigated the influence of specific oligodeoxynucleotide (ODN) analogues on poly(I:C)-induced immuno-mediators, produced via signal transduction pathways different to those for IL-6/IL-8 and in various cell types which may differ in the expression of pattern-recognizing receptors and peculiarities of downstream pathway functioning.

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Materials and Methods

Gingival fibroblasts (GF), endothelial cells (HUVEC) and immature dendritic cells (DC) were obtained from human gingiva, umbilical cord vein and blood monocytes, correspondingly (Mailliard et al. 2004; Cherepanova et al. 2013). Primary and transformed cells (cervical carcinoma (HeLa) and human squamous carcinoma (A431)) were incubated with 100 μg/mL poly(I:C) (Sigma) and/or 1 μM ODN 14 (pctgcatgcatttccttctgcattccagctggat). After treatment, these cells and supernatants were collected for gene expression analysis and IL-6 measurement (Vector-Best, Russia), correspondingly (Cherepanova et al. 2013).

Oligo(dT)18 primer and M-MuLV Reverse Transcriptase (Fermentas, Lithuania) were used for reverse transcription of total RNA. SYBR-Green Real-time PCR was performed using specific forward (f) and reverse (r) primers (5′→3′): IL-6, (f) tctccacaagcgccttcg, (r) ctcagggctgagatgccg; beta-defensin 2 (HBD-2) (f) ctcctcttctcgttcctcttcata, (r) tagggcaaaagactggatgac; interferon beta (hIFN1b) (f) ccaacaagtgtctcctccaaa, (r) gcagtattcaagcctcccatt; GAPDH (f) ttgacggtgccatggaatttg, (r) acggatttggtcgtattgggc. IL-6, HBD-2 and hIFN1b real-time values were normalized to the GAPDH gene mRNA using the $2^{-\Delta\Delta CT}$ method.

Results and Discussion

Poly(I:C) activated IL-6 production in all cell types under investigation. Previously shown inhibition of poly(I:C)-induced interleukins production by specific ODN analogues is also confirmed in HUVEC, Hela and A431 cells, but not in DC (Fig. 20.1a). ODN analogues inhibit poly(I:C)-induced expression of HBD-2 in A431 cells and expression of hIFN1b in GF (Fig. 20.1b). Inhibition of HBD-2 expression confirms the influence of ODN analogues on NFkB and AP-1 transcriptional factors, whereas inhibition of hIFN1b indicates the involvement of IRF3 and IRF7. The data obtained indicate that inhibition occurs at the early stages of the signal transduction cascade: before the recruitment of TRAF6, which activates NFkB via TAB2 polyubiquitination and TRAF3, responsible for IRF3 and IRF7 phosphorylation by TANK-binding kinase 1 (TBK1) (Takeuchi and Akira 2010). The absence of ODN analogue-dependent inhibition of IL-6 production in dendritic cells indicates that the effect is not

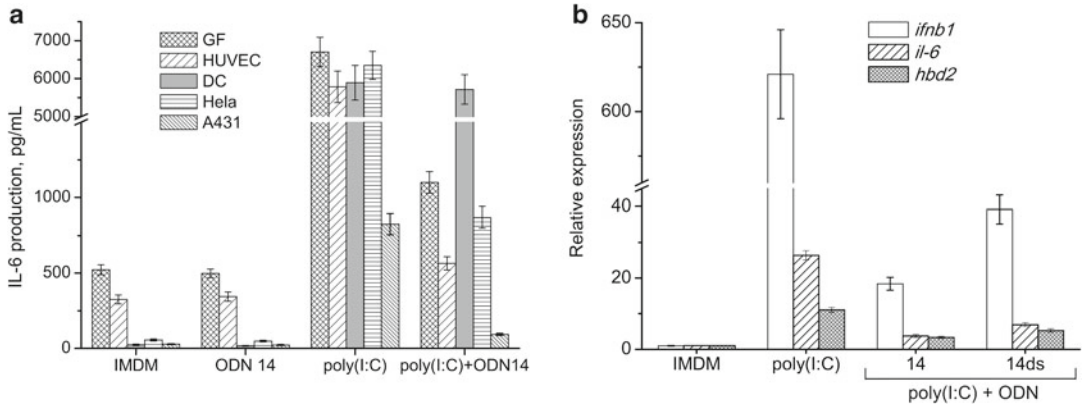

Fig. 20.1 Inhibiting effects of ODN analogue 14 on poly(I:C)-induced activation of primary and transformed cells. (**a**). IL-6 production by different cells 24 h after poly(I:C) and/or ODN analogue treatment. (**b**). IFNb1 expression in GF and IL-6 and HBD2 expression in A431 cells 4 h after treatment. *Error bars* represent standard deviation in each point

universal for all cell types and demonstrates the potentialities of ODN analogues as delicate regulators of immune reactions.

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GC-Rich DNA Fragments and Oxidized Cell-Free DNA Have Different Effects on NF-kB and NRF2 Signaling in MSC

21

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Abstract

It has been established that cell-free DNA circulating in the bloodstream affects cells. The characteristics of cfDNA depend on the physiological state of the organism. As we showed previously, diseases can cause either GC-enrichment of the cell-free DNA pool or its oxidation. Thus, in cases of cerebral atherosclerosis, heart attack and rheumatic arthritis the cell-free DNA pool is GC-enriched and, in the case of cancer, both GC-enriched and oxidized. Herein we investigated the time-dependent effect of oxidized and GC-rich cell-free DNA on NF-kB and NRF2 signaling pathways in human mesenchymal stem cells and showed that they affect cells in different ways. Oxidized DNA drastically increases expression of NRF2 in a short period of time, but the effect does not last long. GC-rich DNA causes a prolonged increase in mRNA levels of NF-kB and NRF2 which lasts 48 and 24 h, respectively.

Keywords

Cell-free DNA • MSCs • Oxidized cell-free DNA • GC-rich cell-free DNA

Introduction

It is a well-known fact that cell-free DNA (cfDNA) circulates throughout the bloodstream, affecting the cells. We investigated various potential biological functions of cfDNA and demonstrated that a GC-rich cfDNA or oxidized cfDNA is a stress signal. As shown previously, a pool of cfDNA consists of GC-rich ribosomal DNA in the serum of patients with some diseases (cere-

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bral atherosclerosis, ischaemic heart disease, heart attack, rheumatic arthritis, cancer) and in some cases, the cfDNA is oxidized (cancer) (Loseva et al. 2012; Ermakov et al. 2013). Our goal was to investigate the effect of these DNA fragments on major signaling pathways.

Materials and Methods

Cells Human mesenchymal stem cells (MSCs) derived from breast adipose tissue were cultivated at 37.°C in AmnioMax C-100 Basal Medium ("Gibco") containing AmnioMax Supplement C-100, 20 mmol/L HEPES ("Pan/Eco") and antibiotics. MSCs were characterized by a number of standard markers (the cultured MSCs expressed major histocompatibility complex molecules (HLA-ABC+) and adhesion molecules (CD44+, CD54 (low), CD90+, CD106+, CD29+, CD49b (low), and CD105); however, these cells were negative for hematopoietic markers (CD34-, CD45-, and HLA-DR-) and the marker CD117 (Dominici et al. 2006)).

DNA Samples Genomic DNA was extracted from human cells using a standard phenol-chloroform extraction method. Hydrolysis by DNase I was then performed to obtain the fragments below 15 kb. Resulting DNA was oxidized by H_2O_2 (Kostyuk et al. 2013). GC-rich DNA fragments were obtained by inserting the CpG rich fragment of rDNA in a vector (pBR322) (Kostyuk et al. 2012). Cell cultures were then incubated with the obtained DNA for different periods of time.

mRNA was isolated from cells using a RNeasy Mini kit (Qiagen, Germany) and treated with DNase I. RNA and DNA concentrations were detected with a fluorescent spectrometer «LS 55» («PerkinElmer», UK). Reverse transcription was performed using a Reverse Transcriptase kit ("Sileks", Russia). The level of gene expression was assessed using RT-PCR. TBP and GAPDH genes were used as internal standards.

Statistical Processing of the results was performed using a standard Statgraphics software package. Mean values were compared using the t – test. All reported results were reproduced at least three times as independent biological replicates.

Results and Discussion

We established that oxidized genomic DNA affects cells the same way as does oxidized cfDNA, so oxidized genomic DNA fragments were used as a model in this work. The source of GC-rich fragments was a GC-DNA-carrying plasmid, which contained a GC-rich insertion, namely, a fragment of human rDNA, in the pBR322 bacterial vector.

The transcription factor NRF2 activates antioxidant response elements – dependent genes that regulate cellular antioxidant defense systems (Pedruzzi et al. 2012). Thus, NRF2 regulates antioxidant and anti-inflammatory cell response (Lau et al. 2008). Oxidized extracellular DNA causes rapid activation of NRF2 signaling pathways in human MSCs (Fig. 21.1). The mRNA level of NRF2 increases sevenfold, and protein levels twofold. Furthermore, oxidized DNA induces a three to fivefold increase in *KEAP1* gene expression after 20 min. The level of *KEAP1* mRNA reaches its maximum (5–6 times) 3 h

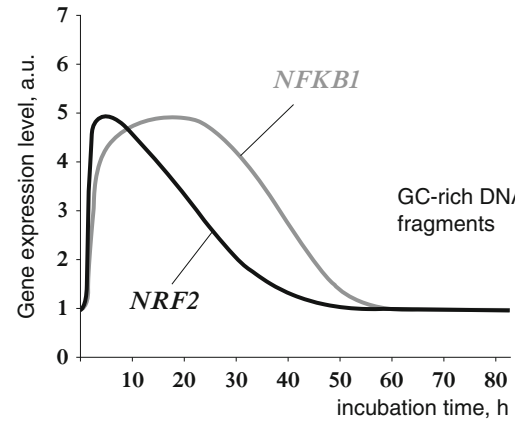

Fig. 21.1 Schematic of dependence of *NRF2* and *NFkB1* level in MSC on time of incubation with GC-rich DNA

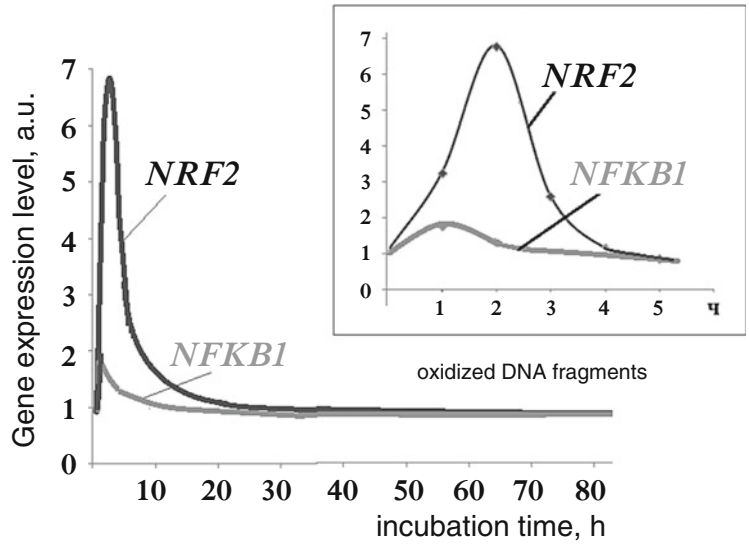

Fig. 21.2 Schematic dependence of *NRF2* and *NFkB1* level in MSC on time of incubation with oxidized DNA

after the addition of oxidized DNA and at the same time, a reduction of *NRF2* expression becomes evident. Moreover, NRF2 activates as a transcription factor by trans-locating to the nucleus and affecting expression of its target gene *SOD1*.

Both transcription and expression of NF-kB have a slight increase 30 min after the addition of oxidized DNA fragments which returns to the control level 30 min later. Moreover, expression of NF-kB target genes *MAP4K4*, *MAP3K1* and *RelA* increases 1.6 – 2-fold. A decrease in the NF-kB mRNA level is consistent with the activation of NRF2 transcription, reaching its maximum after 2 h of incubation.

The addition of GC-rich DNA fragments activates the survival signaling pathways. One of the major survival signaling pathways is the NF-kB pathway. The addition of GC-rich DNA fragments leads to an increment in the transcription of NF-kB signaling pathway genes during 3–24 h after starting the incubation. Expression of *MAP3K1*, *MAP4K4*, *NF-kB1A* and *REL* genes increases 3.5–5.5-fold and expression of *IKBKB*, *RelA*, *NRFKB*, *NF-KB1* and *NF-kB2* – 2–3-fold. GC-rich DNA fragments activate NF-kB which translocates to the nucleus, so increasing the expression of its target-genes *TNFa*, *IL1B*, *IL8*, *IL6* и *IL10*, *ICAM1*, *SELE* and *VCAM1*. Transcription of *NRF2* and *KEAP1* increases 3–24 h after addition. Hence, the GC-rich DNA fragments cause increased expression of NFKB1 along with NRF2 (Fig. 21.2).

Thus, oxidized DNA causes anti-phase regulation of NF-kB and NRF2 signaling pathways in MSC. A similar type of interconnection was earlier described in the literature (Nguyen et al. 2000; Liu et al. 2008). Binding of NF-kB to the NRF2 promoter leads to activation of NRF2 as a transcription factor and suppression of NF-kB (Nair et al. 2008). However, GC-rich DNA fragments cause simultaneous activation of both NRF2 and NF-kB as was shown for multifactor agents such as ROS and LPS (Wakabayashi et al. 2010).

Conflict of Interest Sergeeva V.A., Kostyuk S.V., Ershova E.S., Smirnova T.D., Kameneva L.V., Veiko N.N. state that there are no conflicts of interest.

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22

Evaluation of the State of Transplanted Liver Health by Monitoring of Organ-Specific Genomic Marker in Circulating DNA from Receptor

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Abstract

The evaluation of the transplanted liver health by non-invasive approaches may offer an improvement in early clinical intervention. As transplanted organs have genomes that are distinct from the host's genome, the quantification of the specific DNA of the donated liver in the patient serum will allow us to obtain information about its damage. We evaluated the state of transplanted liver health by monitoring the RH gene in serum circulating DNA (cirDNA) from 17 recipient and donor mismatched for this gene. cirDNA RH gene was quantified by RT- PCR before, at the moment of transplantation (day 0) and during the stay at the intensive care unit. Beta-globin cirDNA was quantified as a general cellular damage marker. Patients were grouped based on clinical outcomes: (A) patients with no complication; (B) patients that accepted the organ but suffered other complications; (C) patients that suffered organ rejection. All patients showed an increased cirDNA levels at day 0 that decreased until patient stabilization. Patients from groups A and B showed low levels of the RH gene cDNA during the follow-up, with an increase of beta-globin gene at the moment of any clinical complication. Patients from group C showed an increase in the RH gene during rejection

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Keywords
Transplantation • Liver • Genomic signature • Organ rejection

Introduction

The health assessment of the transplanted organ to detect the onset of transplant rejection is critical for the long-term survival of the organ. The evaluation of the transplanted liver health by non-invasive approaches may offer a personalized follow-up of the patient and an improvement in early clinical intervention. As transplanted organs have genomes that are distinct from the host's genome, we may find gene polymorphisms (a "genomic signature") to distinguish donated liver DNA in the plasma recipient (Snyder et al. 2011). Thus, the quantification of the specific DNA of the donated organ in the patient serum will allow the obtaining of information about its damage. The aim of this work was to evaluate the state of transplanted liver health by monitoring the donated organ DNA as a biomarker for the early diagnosis of rejection. With this purpose serum cirDNA was monitored for the RH gene in recipients and donors mismatched for this gene

Methods

17 RH-negative patients transplanted with a liver from RH-positive donors were included in the study. cirDNA quantification of the RH gene was performed by RT- PCR before, at the moment of transplantation (day 0) and during the stay in the intensive care unit (ICU). Beta-globin cirDNA levels, a general cellular damage marker, were also quantified. Patients were grouped based on the clinical outcomes: (a) Group A: patients that accepted liver transplantation with no complication; (b) Group B: patients that accepted the male organ but suffer other complications; and (c) Group C: patients that suffered either severe or mild organ rejection.

Results

All patients showed an increase of both RH and beta-globin genes cirDNA levels at day 0 that decreased until patient stabilization. Patients from group A showed low levels of RH gene cirDNA during the follow up. Figure 22.1 shows mean RH gene cirDNA levels during the follow-up of patients with no liver rejection.

Patients from group B showed low levels of RH gene cirDNA during the follow up, but an increase in beta-globin gene levels was observed at the moment of any clinical complication. Patient 11, a representative case from this group, had surgery complications during the first days of transplantation (fever, pleural effusion at day 2 with breathing difficulty at day 5) with variable levels of beta-globin gene cDNA during the stay at ICU. In contrast, RH gene levels did not increase during the 43 days of follow-up. Liver biomarkers were slowly decreasing during this period. On day 23, the patient underwent a jejunostomy for a recanalization of biliary tract occlusion showing only a new increase of beta-globin levels.

Patients from group C suffered either severe or mild liver rejection. Figure 22.2 illustrates a representative patient from this group. This patient suffered a liver rejection at day 7 showing an increase of RH gene levels at this time point. Beta-globin gene showed sustained high levels during the follow-up until stabilization at day 18. An increase of glutamic-pyruvate transaminase levels was also observed at the day of organ rejection.

Discussion

The monitoring of serum cirDNA of RH gene in recipients and donors mismatched for this gene might be useful as an initial approach for the

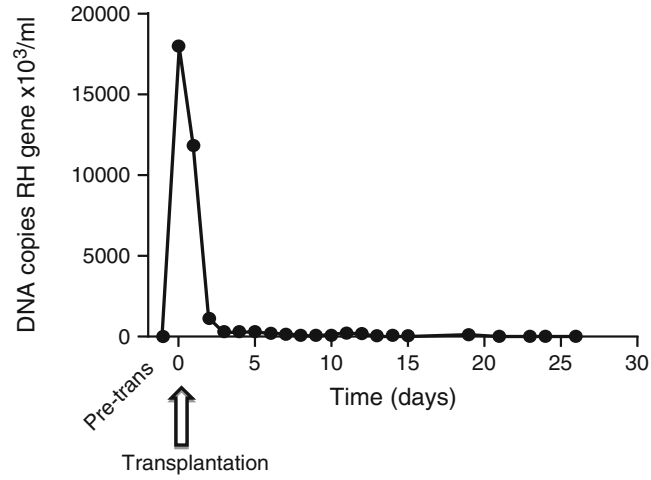

Fig. 22.1 Mean RH gene cDNA levels of patients with no liver rejection

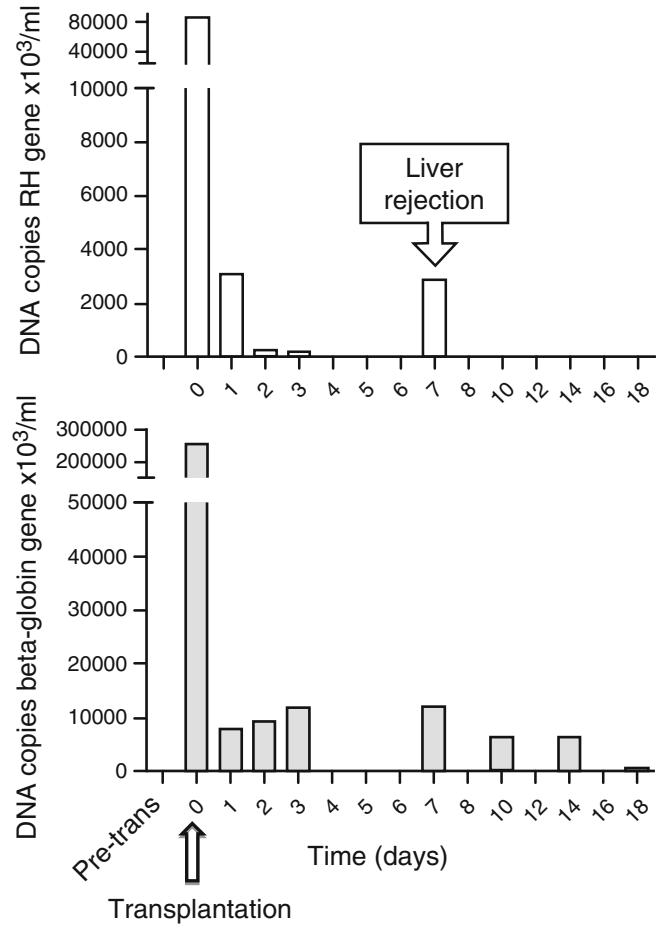

Fig. 22.2 RH gene and Beta-globin gene cDNA levels of a patient presenting with liver rejection

evaluation of the transplanted liver health. In this way, even when a patient suffered a mild organ rejection, RH gene levels were clearly increased at this time point. Supporting these results, we also reported in a previous study the detection of the SRY gene in the subset of patients where the

donor was male and the recipient female (Macher et al. 2014).

On the other hand, beta-globin gene cDNA levels might be used for the evaluation of the general clinical patient state. Thus, when patients presented clinical complications, variable levels of beta-globin gene cDNA were observed. However, as the liver was not damaged, RH gene levels did not increase during the complete follow up.

In conclusion, our results show that specific donor-derived cirDNA may be quantified in the serum of organ transplant recipients and might be used as an indication of graft injury.

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Vesicular and Extra-Vesicular RNAs of Human Blood Plasma

23

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Abstract

Human blood contains a great variety of membrane-covered RNA carrying vesicles which are spherical or tubular particles enclosed by a phospholipid bilayer. Circulating vesicles are thought to mediate cell-to-cell communication and their RNA cargo can act as regulatory molecules. In this work, we separated blood plasma of healthy donors by centrifugation and determined that vesicles precipitated at 16,000 g were enriched with CD41a, marker of platelets. At 160,000 g, the pellets were enriched with CD3 marker of T cells. To characterize the RNA-content of the blood plasma sub fractions, we performed high throughput sequencing of the RNA pelleted within vesicles at 16,000 g and 160,000 g as well as RNA remaining in the vesicle-free supernatant. We found that blood plasma sub fractions contain not only extensive set of microRNAs but also fragments of other cellular RNAs: rRNAs, tRNAs, mRNAs, lncRNAs, small RNAs including RNAs encoded by mtDNAs. Our data indicate that a variety of blood plasma RNAs circulating within vesicles as well as of extra-vesicular RNAs are comparable to the variety of cellular RNA species.

Keywords

Circulating RNA • Exosomes • Microvesicles • Next generation sequencing • SOLiD™

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Introduction

Membrane-covered vesicles such as exosomes, microvesicles, apoptotic bodies as well as extracellular ribonucleoproteins are secreted by different types of cells, their RNA reflecting the physiological state of the donor cells. Circulating vesicles being captured by a recipient cell induce a cellular response, alter physiological processes and are hereby involved in cell-to-cell signaling. To date, studies have focused on the microRNA content of extracellular vesicles while it is known that cells actively secret RNA of other classes (Yáñez-Mó et al. 2015).

In the present work we used SOLiD high throughput sequencing technology, in combination with an approach, described previously (Semenov et al. 2012) to characterize RNome of human blood plasma extracellular vesicles and ribonucleoproteins.

Materials and Methods

Blood samples of healthy donors were sequentially centrifuged at 1200 g, followed by 16,000 and 160,000 g. 16,000 and 160,000 g pellets were analyzed using JEM 1400 (Jeol, Japan). Staining with antibodies to CD41a, CD3, CD34, CD79a, CD63 (eBioscience, USA) and flow cytometry using FACS Canto II (Becton Dickinson, USA) were performed. The total RNA of blood plasma sub fractions was extracted with TRIzol. cDNA libraries construction, sequencing with high-throughput SOLiD 5500xl platform and data analysis were performed as previously described (Semenov et al. 2012). SOLiD sequencing was performed in the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk).

Results and Discussion

We pooled blood plasma of healthy donors with sequential centrifugation steps and obtained pellets precipitated at 16,000 g, 160,000 g as well as a vesicle-free supernatant. Using scanning electron microscopy it was shown that both 16,000 g and 160,000 g pellets were enriched with exosomal-like membrane-covered structures of 40 – 100 nm. With fluorescent antibody staining and flow cytometry analysis it was determined that plasma vesicles, pelleted at 16,000 g were enriched with the CD41a marker for platelets and mega-karyocytes 69.37 ± 9.02 % (ave ± SD) (Fig. 23.1a). Fractions pelleted at 160,000 g were enriched with the CD3 marker for T-cells 54.37 ± 30.44 % (Fig. 23.1b). The CD79a B-cell marker and CD63 common exosome marker were also detected in 5.0 ± 4.0 % and 51.4 ± 12.8 % of the 160,000 g blood plasma vesicles, respectively.

We extracted total RNA from blood plasma 16,000 g and 160,000 g pellets as well as of the vesicle-depleted supernatant and constructed SOLiD cDNA-libraries. DNA libraries were sequenced with SOLiD 5500xl platform and 33.7 millions of reads were obtained. It was determined that 16,000 g and 160,000 g pellets as well as particle-depleted supernatant contained fragments of all known types of cellular RNAs: rRNAs, tRNAs, mRNAs, the variety of small and long non-coding RNAs including microRNAs, Y-RNAs, vault-RNAs. Fragments of mitochondrial transcripts were also detected in all plasma sub fractions with prominent enrichment in the 16,000 g pellets (Table 23.1).

Previously, Nolte-'t Hoen et al. (2012) showed that shuttle RNA released during murine DC-T cell interactions were enriched with non-coding RNA species except miRNAs and lncRNAs, wherein the most represented groups were rRNAs and vault-RNAs (~30 %). Huang et al. (2013) determined that human blood plasma vesicles, precipitated with ExoQuick reagent were enriched with microRNAs (76.2 %) and also contained almost all cellular RNA species, including rRNAs (9.16 %) and tRNAs (1.24 %). Observed differences in the contribution of particular RNA species could be related to several points: the biological origin of the vesicles, RNA extraction protocol-based bias and the strategies used for computational sequencing data analysis.

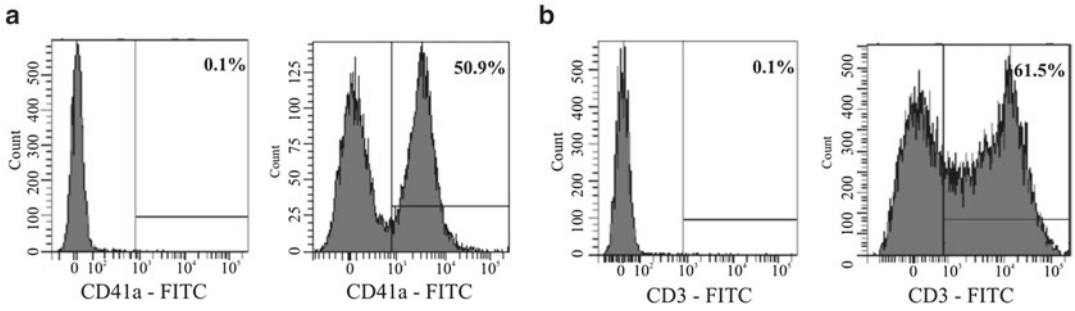

Fig. 23.1 Flow cytometry analysis of human blood plasma pellets. (**a**) – The content of vesicles in 16,000 g pellet positive for CD41a. (**b**) – The content of vesicles in 160,000 g pellet positive for CD3. *Left* diagrams demonstrate the data of unstained particles (**a**, **b**)

Table 23.1 The distribution of RNA species in human blood plasma subfractions

			The contribution of RNA species, % of all mappable counts							
Blood samples origin	Blood subfraction	Total number of reads	mRNAs	rRNAs	tRNAs	Noncoding RNAs (RefSeq)	Transcribed genomic repeats	mtRNAs	Noncoding RNAs (Non RefSeq)[a]	Unclassified
Healthy donors	16,000 g vesicles	7,888,961	15.3	40.2	0.41	6.67	0.92	17.78	18.72	8.0
	160,000 g vesicles	12,567,476	25.2	20.3	0.5	3.7	0.52	2.81	46.99	8.2
	Vesicle-free plasma	13,196,829	21.1	29.4	1.05	5.39	1.65	7.57	33.78	8.4

[a] fragments of long non-coding RNAs not annotated in human RefSeq and intronic sequences

Both our results and published data indicate that RNome of secreted membrane-covered vesicles as well as RNome of extracellular vesicle-free complexes are not restricted to the particular RNA species, such as microRNAs, but rather exhibit a complexity comparable to the complexity of cellular RNAs.

Acknowledgements We wish to thank Professor Ryabchikova E.I. D.Biol.Sci. for microscopy investigations. We also thank Kurilshchikov A.M. for the support in bioinformatics. The work was supported by the RFBR grants 16-04-01457 A and the Interdisciplinary Integration Project of SB RAS № 84 (2012–2015).

Conflict of Interest The authors declare no conflict of interest.

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24 Artificial Analogues of Circulating Box C/D RNAs Induce Strong Innate Immune Response and MicroRNA Activation in Human Adenocarcinoma Cells

Grigory A. Stepanov, Julia A. Filippova, Anna A. Nushtaeva, Elena V. Kuligina, Olga A. Koval, Vladimir A. Richter, and Dmitriy V. Semenov

Abstract

Fragments of small nucleolar RNAs (snoRNAs) were found among various non-coding RNAs (ncRNAs) circulating in human blood. Currently, the function of such cell-free sno-derived-RNAs is not clearly defined. This work is aimed at identifying regulatory pathways controlled by extracellular snoRNAs. In order to determine the molecular targets and pathways affected by artificial snoRNAs, we performed Illumina array analysis of MCF-7 human adenocarcinoma cells transfected with box C/D RNAs. The genes related to the innate immune response and apoptotic cascades were found to be activated in transfected cells compared with control cells. Intriguingly, the transfection of MCF-7 cells with artificial box C/D snoRNAs also increased the transcription of several microRNAs, such as mir-574, mir-599 and mir-21. Our data demonstrated that extracellular snoRNAs introduced into human cells may function as gene expression modulators, with activation of microRNA genes being one of the regulatory mechanisms.

Keywords

Circulating RNAs • Small nucleolar RNAs • Innate immune response • MicroRNAs

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Introduction

The family of small nucleolar RNAs (snoRNAs) consists of two major classes, namely box C/D and box H/ACA snoRNAs; these non-coding RNAs (ncRNAs) complexed with specific nucle-

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olar proteins direct post-transcriptional nucleotide modification of eukaryotic ribosomal RNAs: 2′-O-methylation and pseudouridylation, respectively (Cavaille et al. 1996; Ganot et al. 1997). To date, it has been established that snoRNAs have many features going beyond the nucleolus as a compartment of their major activity. Some snoRNAs are involved in control of post-transcriptional nucleotide modification and pre-mRNA splicing (Kishore and Stamm 2006; Vitali et al. 2005). Box C/D RNAs and box H/ACA RNAs alongside with other ncRNAs were reported to serve as precursors of smaller RNAs that can regulate alternative splicing, translation efficiency of complementary mRNAs and their stability (Falaleeva and Stamm 2013; Kishore et al. 2010; Ono et al. 2011; Brameier et al. 2011). A few full-length snoRNAs and snoRNA-derived microRNA-like fragments can be transported from nucleus to cytoplasm and secreted into the extracellular space. Thus, they can serve as mediators of intracellular and intercellular signals (Michel et al. 2011; Holley et al. 2015; Semenov et al. 2012). Some circulating snoRNAs have been proposed for use as diagnostic biomarkers (Liao et al. 2010; Stepanov et al. 2015). Investigation of snoRNA functioning mechanisms seems to be promising for diagnostic and therapeutic approaches and is of current interest for both applied and fundamental research. It still remains elusive as to how extracellular snoRNAs penetrating into human cells can affect intracellular processes.

Methods

Artificial analogues of box C/D RNAs were obtained using *in vitro* transcription by T7 RNA polymerase as described earlier (Stepanov et al. 2013; Stepanov et al. 2012). Pre-miRNA and mRNA levels were assessed using BioMaster RT-PCR – SYBR Blue Mixes (Biolabmix, Novosibirsk, Russia) with specific primers for IFIT3: IFIT3_1: 5′-GGCAGACAGGAAGACTT CTG-3′ and
IFIT3_2: 5′-TTTCTGCTTGGTCAGCATGT-3′;
for pre-miR-21:
pre_mir21_L: 5′-CTTATCAGACTGATGTTGA CTG-3′ and
pre_mir21_R: 5′-TGTCAGACAGCCCATCGA CTG-3′.

Microarray analysis was performed using Illumina HT-12 array by "Genoanalytica" (Moscow, Russia) followed by functional analysis of changed genes with Cytoscape v. 2.8.0 (ClueGO plugin v. 1.4) (Shannon et al. 2003).

RNA sequences: RNA7: 5′-GGGUGCAGAU GAUGUAAAAUAGCGACGGGCGGUG CUGAGAGAUGGUGAUGACAAAU GAAAACACUUUCAAUCUGAUGCA-3′
RNA5: 5′-GGGUGCAGAUGAUGUAAAAUAG CGACGGGCGGUGCUGAGAGAU GGUGAUGAACGACGGUCUAAA CCCUGAUGCACCC-3′

(Presented in the tables and figures are the data obtained for RNA5; similar results were obtained for RNA7).

Control RNA: 5′-GGGUCGAUAAGCUUGAUA UCGUAUCACAGCAAAAUAAACAAU CAGUAAAGGGACAACCUACAAA AUGGCAGAGAAUAUUUGCAAAC UAUUCAUCUGCAAAGCGGUUAA UAUCCAGAAUACACAGGGAAUUC AAUUCCUGCAGCCCGGGGGAUCCA-3′.

Results and Discussion

Box C/D snoRNA analogues holding canonical elements of human U24 snoRNA (SNORD24) have been synthesized. Artificial RNAs had novel guide sequences, so these RNAs were targeted at predetermined nucleotides of different types of cellular RNAs (Stepanov et al. 2012, 2013). Here, we analyzed the transcriptome of human adenocarcinoma MCF-7 cells transfected by artificial box C/D RNAs using Illumina microarrays and found that snoRNA analogues induced cellular

Table 24.1 The most numerous functional groups of genes activated by artificial box C/D RNAs

GO-term	Number of genes	pValue
GO:0045087: innate immune response	43	3.48E-34
GO:0034097: response to cytokine stimulus	36	1.92E-31
GO:0016032: viral process	33	3.44E-24
GO:0009615: response to virus	31	1.10E-33
GO:0019221: cytokine-mediated signaling pathway	31	1.89E-30
GO:0071345: cellular response to cytokine stimulus	31	3.26E-27
GO:0034340: response to type I interferon	27	6.86E-44
GO:0051607: defense response to virus	27	1.29E-31
GO:0060337: type I interferon-mediated signaling	26	6.78E-42
GO:0071357: cellular response to type I interferon	26	6.78E-42

innate immune response. Functional hierarchical clustering of up-regulated genes revealed enrichment in innate immune response groups (Table 24.1). The up-regulated gene groups included interferon-induced genes such as DDX58, MYD88, OAS, IFIT- (IFIT1-3) and IRF- (IRF-1,7) families and pro-apoptotic genes CASP7, TNFSF10 and IFIT3. Comparative analysis of the activated gene sets with VISTA Browser (www.genome.lbl.gov) confirmed an increase in NF-kB- and IRF8/9-dependent gene levels (Table 24.2).

The observed immune-stimulated effect was specific only for box C/D RNA. Control RNA did not cause such an effect (Fig. 24.1). It was assumed that 5′-triphosphate and base-paired structures near the 5′-end of box C/D RNAs were

Table 24.2 Transcription factors controlling groups of up-regulated genes in MCF-7 cells after transfection with artificial box C/D RNAs

TF Vista ID	TF	$-\log_{10}$(p-value)	Gene group
288	ICSBP (IRF8)	2.3941	*SP100, ISG15, TRIM56, IFIH1, IFI6, HLA-A, IFIT3, SOCS1, TDRD7, BST2, IFI44, XAF1*
306	ISGF-3 (IRF9)	2.5861	*ISG15, IFIH1, IFIT3, SOCS1, BST2, IFI44*
390	NF-kB (p50:p65)	1.5402	*CFB, ISG20, SOCS1, IRF1, CCL5*

the key features that provide the effect given (data not shown); this is in accordance with the data on the ability of such structural features to synergize to trigger the induction of signaling competent RIG-I multimers (Schmidt et al. 2009).

Intriguingly, the list of RNAs with increased expression levels contains several miRNAs including mir-574, mir-599 and mir-21. Further bioinformatic analysis allowed us to establish that the box-C/D-RNA-dependent activation of these miRNAs suppressed an extensive group of cellular RNAs, including mRNA and ncRNA, via suppression of transcriptional factor synthesis. An increase in the level of mir-21 precursor in MCF-7 and MDA-MB-231 adenocarcinoma cells was confirmed by RT-PCR analysis (Fig. 24.2a). Activation of innate immunity in transfected cells was confirmed by the changes in IFIT3 mRNA level (Fig. 24.2b).

In conclusion, we propose that extracellular snoRNAs penetrating into human cells can function as modulators of vital gene expression including regulation of microRNA levels. Some microRNAs such as mir-21, mir-1228 and mir-599 may play an important role in the innate immune response in human cells.

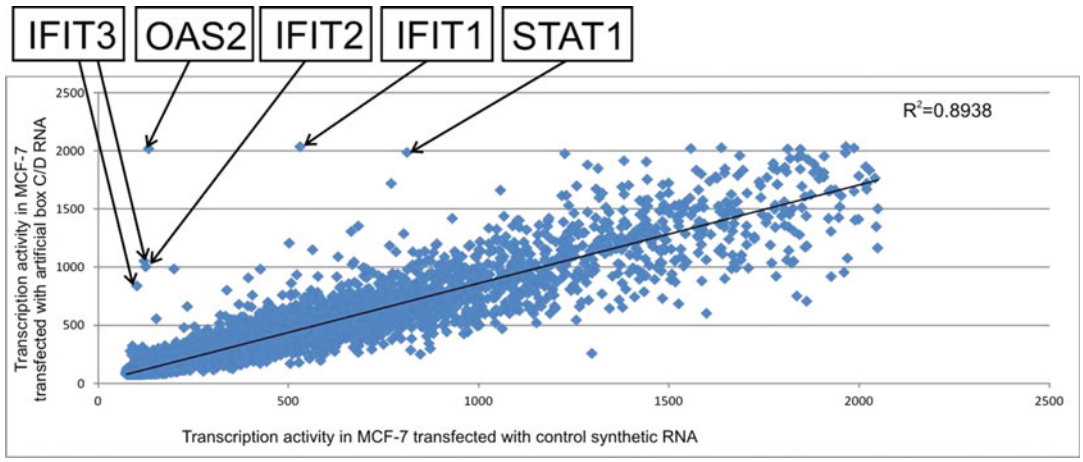

Fig. 24.1 Comparison of gene expression profile in human cells transfected with box C/D RNA5 and control RNA

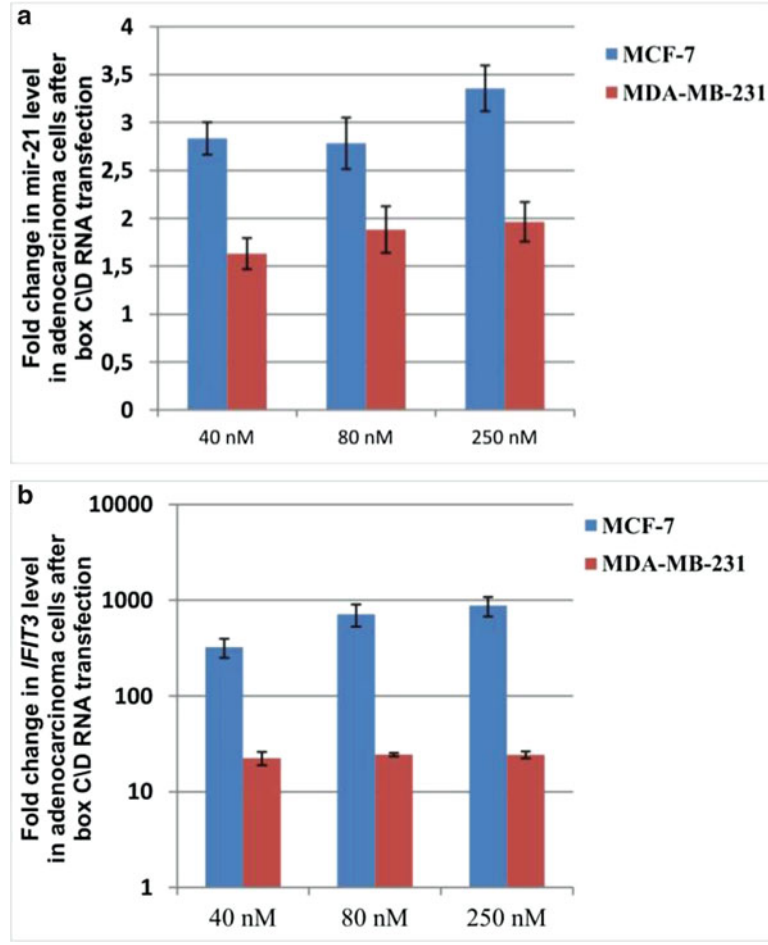

Fig. 24.2 Changes in transcription activity of genes encoding mir-21 (**a**) and IFIT3 (**b**) in adenocarcinoma MCF-7 and MDA-MB-231 cells upon transfection with box C/D RNA

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Conflict of Interest None.

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Multiple Ways of cfDNA Reception and Following ROS Production in Endothelial Cells

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Anna Yu. Alekseeva, Larisa V. Kameneva, Svetlana V. Kostyuk, and Natalia N. Veiko

Abstract

Oxidized cell-free DNA acts as a stress signal molecule and triggers an adaptive response in human cells. Various membrane DNA recognizing receptors are known as potential sensors for such DNA fragments. In order to clarify which of these sensors are able to interact with cfDNA fragments, circulating in human blood flow in heath and disease, we studied the influence of various cfDNA types on endothelial cells. We incubated these fragments at a physiologically optimal concentration with HUVEC cells for 3–24 h and detected the expression of either TLR9 or AIM2, RIG1 and STING receptors at mRNA and protein levels. We estimated that the activation of both TLR9 and other types of intracellular receptors initiates stress signaling in the endothelium independently. Signal transduction through these receptors activates NOX4 as the main source of ROS production in HUVECs.

Keywords

HUVECs • Cell-free DNA • DNA sensors • ROS • Cardiovascular diseases

Introduction

Heart attack and other cardiovascular diseases promote high levels of cell-free DNA (cfDNA) in the blood plasma (Wang et al. 2015). In many studies, it has been shown that the pool of cfDNA fragments circulating in blood of people with acute heart attack, stroke or inflammatory diseases are enriched with CG fragments which are also highly oxidized (Ermakov et al. 2013). The endothelial cells that line blood vessels can recognize these cfDNA fragments and evoke the mechanisms of oxidative stress in cell cultures (Alekseeva et al. 2011). Cell free DNA may enter the cell and even its nucleus (Gahan 2015). However, cfDNA recognition resources in HUVECs and the mechanics of the reactive

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oxygen species (ROS) production as one branch of the cell response to the cfDNA are largely unknown. In the case of other cell types, there are enough data concerning the role of NADPH-oxidase (NOX) in the production of ROS (Altenhofer et al. 2012). In our study, we sought to explore the mechanisms of possible interaction of the cfDNA with endothelial cells as well as the origin of ROS production.

Materials and Methods

Cell Culture

Human umbilical vein cells (HUVECs of passages 2–4) were cultured in 4 ml slide-flasks (Corning-Costar, UK) under previously described conditions (Alekseeva et al. 2011).

CfDNA Samples

HUVECs were treated with different synthetic cfDNA fragments as well as the cfDNA extracted from blood plasma of both healthy individuals (cfDNAh) and people with a heart attack (cfDNAstr). Human cfDNA was isolated from peripheral blood plasma; genome DNA (gDNA) was isolated from HUVECs by organic solvent extraction as described previously (Alekseeva et al. 2011). In order to obtain oxidized DNA (DNAoxy), gDNA was treated with 300 mM H_2O_2 in the presence of 10 μM Fe^{2+} and 10 μM EDTA for 30 min at 25 °C in the dark. We used a CpG-rich fragment of rDNA (9504 bp, the site between 515 and 5321 in accordance with HSU13369, GeneBank) in the pBR322 vector to obtain CG-rich DNA (CG-DNA). All DNA samples were subjected to a standard purification procedure (Kostyuk et al. 2012). Cell-free DNA concentration was determined by a fluorescence-based method (Szpechcinski et al. 2008).

Blocking of TLR9 Receptors

The ligand/receptor interactions were blocked for 30 min at 37 °C prior using either 3 μg/ml of a non-selective TLR and p38-pathway inhibitor – SB203580 (InvivoGen, USA) or 2 μg/ml chloroquine (Boots Company PLC, UK).

Quantification of mRNA Levels for TLR9, NOX4, AIM2, RIG1, STING and TBP

The gene expression levels were evaluated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) performed on the StepOnePlus TM Real-Time PCR System (Applied Biosystems, USA) using the SYBR Green dye. Total mRNA was isolated from cells using RNeasy Mini kit (Qiagen, Germany).

Fluorescent Microscopy and Flow Cytometry Analysis

Immunostaining was performed as previously described using FITC-conjugated or PE-conjugated antibodies (Abcam) to TLR9 and NOX4 (Alekseeva et al. 2011; Kostyuk et al. 2012). Cell images were obtained using the AxioScope A1 microscope (Carl Zeiss). Flow cytometry analysis was performed at CyFlow Space (Partec, Germany).

Statistics

All reported results were reproduced at least three times in independent experiments. The figures show the averaged data and the standard deviation (SD). The significance of differences was analyzed using the parametric *t*-test (for comparisons when $n > 30$) or the non-parametric Mann-Whitney tests (for comparisons with $n < 30$).

Results

During the experiment, we detected a 2–5 fold increase of the *TLR9* mRNA levels in endothelial cells treated by various DNA fragments after 3 h. Chloroquine significantly ($p<0.01$) reduced this effect (Fig. 25.1a). After a 3-h incubation with CG-rich oxidized DNA fragments (50 ng/ml) as well as with cfDNA fragments from blood of patients with stroke (cfDNAstr), we observed the level of the *TLR9* expression to be more than four times higher when compared to control (Fig. 25.1b–d).

To address the mechanism of the oxidative stress formation, which is known to occur in endothelial cells after the cfDNA treatment (Kostyuk et al. 2014), we also measured *NOX4* expression. We mentioned a positive correlation between rise in *NOX4* mRNA expression and the amount of ROS detected in HUVECs. The mRNA levels of the *NOX4* gene and NOX production, both with or without chloroquine, remained 2–6 times higher after a 3-h incubation in the presence of the synthetic cfDNA and cfDNAstr fragments (Fig. 25.2 a, b). Only SB203580 blocking the TLR and p38 signal pathways inhibited (**$p<0.01$, *Mann-Whitney u-test*) the effects of the highly oxidized cfDNA on both oxidative stress and *NOX4* expression (Fig. 25.2a). Consequently, the influence of cfDNAox on the endothelium, implemented through ROS production in HUVECs, turns out to be initiated not only by a TLR-cascade.

These results led us to search for other potential cfDNA receptors among the DNA and RNA recognition proteins, like *AIM2* and *RIG1*. We detected also a significant rise in the *AIM2* and *RIG1* expression. Alternatively, the stimulator of interferon genes (*STING*) is an important regulator of the innate immune response to cytoplasmic DNA (Gehrke et al. 2013). The results of the qRT-PCR profiling of the *STING* mRNA in HUVECs exposed to various types of DNA also displayed up to a 10-fold increase in the DNA-treated cells in comparison to the untreated ones (Fig. 25.3).

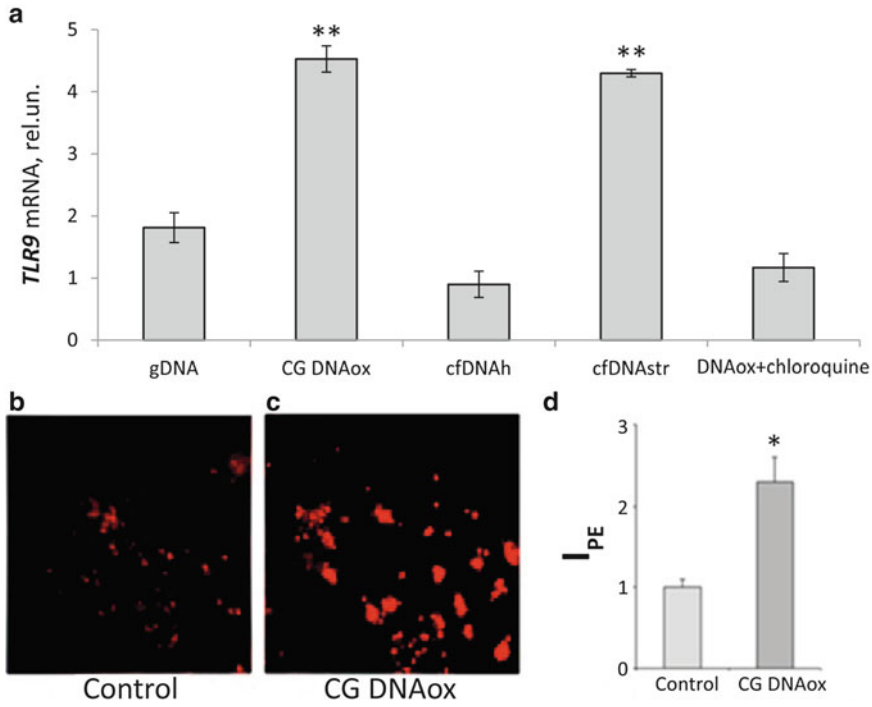

Fig. 25.1 (**a**). Changes in the *TLR9* mRNA levels, with reference to the TBP gene (qRT-PCR). (**b**), (**c**). TLR9 protein expression in HUVEC cells under the influence of various DNA fragments (Fluorescent microscopy, magnification×48). (**d**). TLR9 protein expression in HUVEC cells under the influence of various DNA fragments (Flow cytometry, I_{PE}- average values of fluorescence intensity in a single cell, for 100 cells). Quantitative data are presented as a mean±standard deviation (*p<0.05, **p<0.01, Mann-Whitney *u*-test and Student's *t*-test)

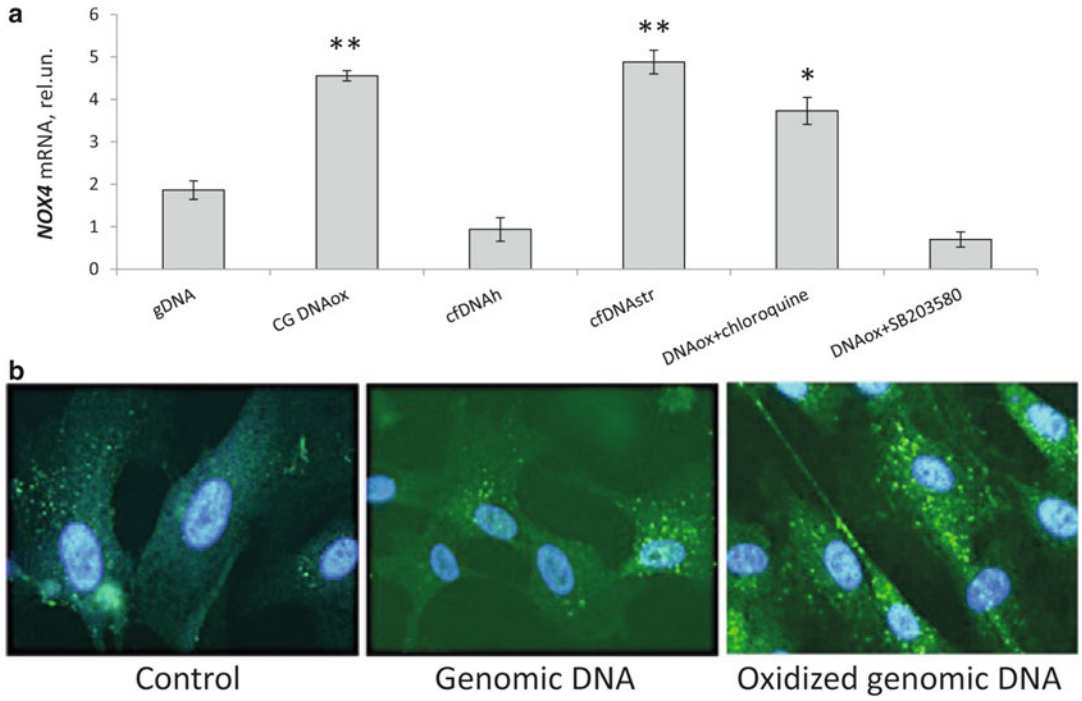

Fig. 25.2 (**a**) Changes in the *NOX4* mRNA levels in HUVEC cells, with reference to the TBP gene (qRT-PCR, quantitative data are presented as a mean ± SD (*p < 0.05, **p < 0.01, Mann-Whitney *u*-test). (**b**) NOX4 protein expression in HUVEC cells under the influence of various DNA fragments at a concentration of 50 ng/ml (fluorescent microscopy, magnification × 100)

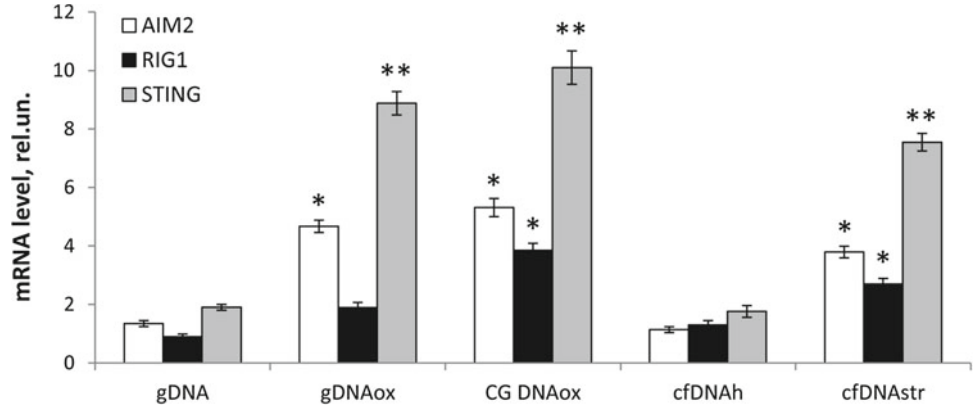

Fig. 25.3 Results of the qRT-PCR profiling of the *AIM2*, *RIG1* and *STING* mRNA in HUVECs exposed to various types of DNA during a 3-h incubation. The data are presented as a mean ± SD (*p < 0.05, **p < 0.01, Mann-Whitney *u*-test)

Our results may point to new yet unidentified branches of molecular cascade that are triggered by the cfDNA.

Discussion

Various sensors of nucleic acids have been identified in endothelial cells. These sensors are capable of binding the cfDNA fragments that in turn induce different types of cell signals that significantly affect the cell status and stimulate the development of an adaptive response, including ROS production. We showed that fragments of the cfDNA influence the endothelial cells through binding to the DNA recognition receptors, such as TLR9 along with other nucleic acid recognition sensors that trigger intracellular stress cascades. Multiple ways of the cfDNA sensing by endothelial cells may indicate new ways of therapy in patients who suffer from cardiovascular, autoimmune and other diseases that induce a massive release of the cfDNA in the blood stream.

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Conflict of Interest A.Y. Alekseeva, L.V. Kameneva, S.V. Kostyuk and N.N. Veiko state that there are no conflicts of interest.

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Protein Content of Circulating Nucleoprotein Complexes

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Abstract

In the current study we have investigated the protein content of blood plasma deoxyribonucleoprotein complexes. The complexes were isolated using affinity chromatography with immobilized polyclonal anti-histone antibodies. Proteins were separated by SDS PAAGE and identified by MALDI-TOF mass-spectrometry. 111 and 56 proteins (excluding histones), respectively, were identified with a good score in deoxyribonucleoprotein complexes of healthy females and breast cancer patients. However, only four of these proteins were found in 30 % of all samples. Fourteen proteins previously described as tumor specific proteins were found in cancer patients whereas not one of them was found in healthy individuals. The data obtained demonstrate the involvement of different cellular and extracellular proteins in circulating cell-free DNA.

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Keywords
Circulating DNA • Nucleoprotein complexes • DNA-binding proteins • MALDI TOF mass spectrometry • Plasma

Introduction

It's known that cell-free DNA (cfDNA) circulated in bloodstream being packed into membrane coated structures like apoptotic bodies or complexes with biopolymers like histones or DNA-binding plasma proteins (Bryzgunova and Laktionov 2014). Many serum, connective tissue and cellular proteins were shown to bind to DNA, but only few were shown to bind to cfDNA in circulating blood (Butler et al. 1990; Holdenrieder and Stieber 2004; Laktionov et al. 1999). Proteins complexing with cfDNA can interfere with the lifetime of cfDNA in the blood, its clearance and tissue distribution as well as reflecting the cfDNA origin. Thus, identification of the proteins involved in cfDNA circulation in blood is needed for a general description of the cfDNA phenomenon and can potentially bring helpful information concerning the enrichment of cancer-specific cfDNA.

In the current study we have investigated the protein content of deoxyribonucleoprotein complexes (DNPC) circulating in the blood plasma of healthy females (HFs) and primary breast cancer patients (BCPs).

Materials and Methods

Blood samples of previously untreated BCPs (n = 5, T1-2N0M0, estrogen and progesterone receptor positive infiltrative ductal carcinoma) and HFs (n = 5, without any female diseases of the reproductive system) were enrolled in the study in accordance with current rules. Blood cells were removed by successive centrifugation at 350 g for 15 min and then at 1200 g for 20 min. Blood plasma DNPC were isolated by affinity chromatography with antihistone antibodies immobilized on Sepharose 6B; the proteins were separated by 10–20 % SDS-PAGE and identified by mass spectrometry as described earlier (Tamkovich et al. 2015). Protein concentration in DNPCs was measured by NanoOrange Protein Quantification kit as recommended by the manufacture (Molecular Probes, USA). The molecular function of identified proteins were described either using the InterPro database or predicted by Expert System PolyOmics.

Results and Discussion

The approach of DNPC isolation used in the current study mainly isolated nucleosomes as confirmed by the DNA content of the isolated DNPS (mainly 170–180 bp DNA) (Tamkovich et al. 2015). Comparative analysis of DNPC protein concentration isolated from the blood of HFs and BCPs do not show significant differences (Mann–Whitney *U*-test) (Fig. 26.1a).

One hundred and eleven and 56 additional proteins (excluding histones) were identified by mass-spectrometry with a score exceeding 56 in the DNPC of HFs and BCPs, respectively. However, only 22 proteins were found in both patient groups (Fig. 26.1b). Analysis of 93 proteins was made using InterPro database localization and molecular functions. It was found that 40 % of the identified proteins bound to nucleotides and nucleic acids (Fig. 26.2a), but the localization of the main part these proteins is unknown (Fig. 26.2b).

The identified proteins were analyzed using Expert System PolyOmics algorithm predicting DNA-binding sites from amino acids sequence. Eight multidomain Zinc fingers, two Zinc fingers and five Leucine zippers were found in the DNPS of HFs and BCPs (Table 26.1).

The most universal proteins in the DNPC are Homeobox protein Hox-C5, Probable G-protein

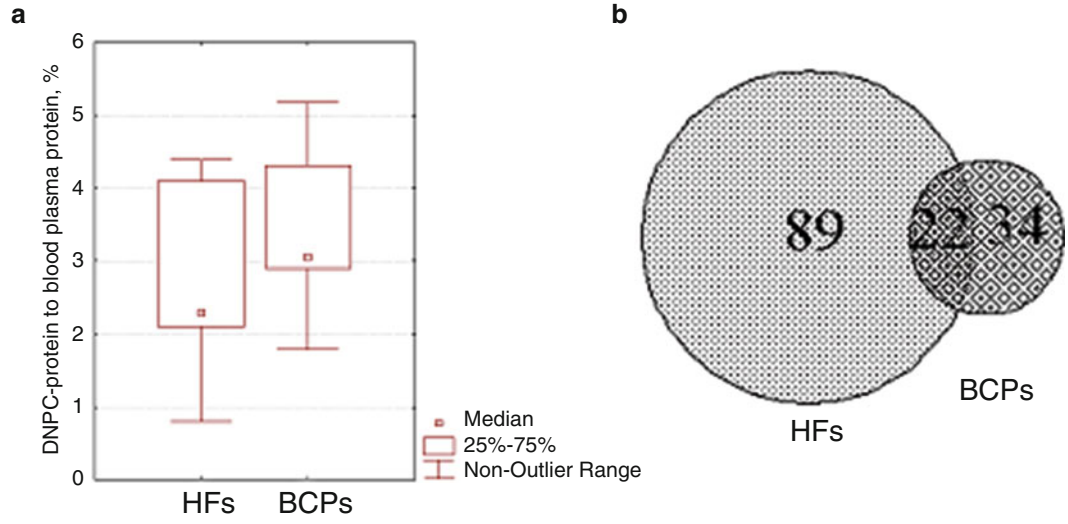

Fig. 26.1 Proteins of blood plasma DNPC from HDs and BCPs. (**a**) DNPC-protein versus total blood plasma protein, %; (**b**) Venn-Euler diagram of proteins from plasma DNPC

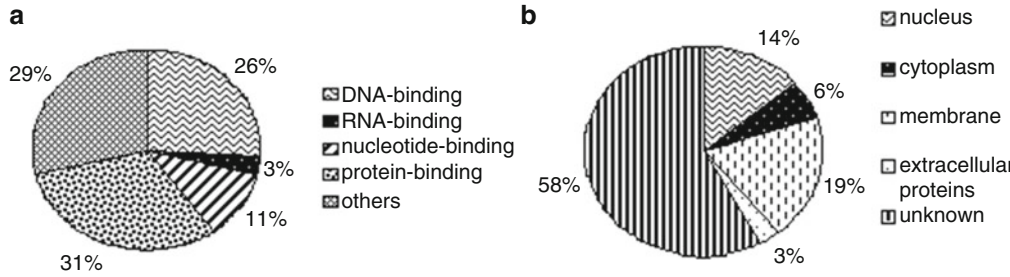

Fig. 26.2 Function and intracellular localization of DNPC proteins

Table 26.1 DNA-binding motifs in DNPC proteins

DNA-binding motif	Protein
Multidomain Zinc fingers	Protein SMG7
Cys2His2	Early growth response protein 4
	PHD finger protein 1
	Vascular endothelial growth factor A
	E3 ubiquitin-protein ligase TRIM68
	Mitogen-activated protein kinase kinase 14
	Ubiquitin carboxyl-terminal hydrolase 22
	Rho guanine nucleotide exchange factor 9
Zinc fingers	E3 ubiquitin-protein ligase TRIM63
Cys-X2-CysX17-Cys-X2-Cys	E3 ubiquitin-protein ligase ZNF645
Leucine zippers	Transcription factor AP-1
N-X7-R-X9-L-X6-L-X6-L	cAMP-responsive element modulator
	Cyclic AMP-dependent transcription factor ATF-1
	Transcription factor jun-D
	Transcription factor MafB

Table 26.2 Main DNPC proteins

Protein	HFs (of 5)	BCPs (of 5)
Homeobox protein Hox-C5	4/5	1/5
Probable G-protein coupled receptor 22	3/5	3/5
Insulin-degrading enzyme	1/5	3/5
ß-1,3-glucosyltransferase	0/5	2/5
G1/S-specific cyclin-E2	0/5	2/5
Noelin-3	0/5	2/5
Rho GTPase-activating protein 30	0/5	2/5
NADP-dependent malic enzyme	0/5	2/5
Protein asteroid homolog	0/5	2/5
Peroxisomal trans-2-enoyl-CoA reductase	0/5	2/5
Acyl-coenzyme A thioesterase 11	0/5	2/5
Autoimmune regulator	0/5	2/5
SHC SH2 domain-binding protein 1-like protein	0/5	2/5
Chondroitin sulfate N-acetylgalactosaminyltransferase 1	0/5	2/5
Protein Mdm4	0/5	2/5
Rho guanine nucleotide exchange factor 9	0/5	2/5
Neuroplastin	0/5	2/5

coupled receptor 22 and Insulin-degrading enzyme (found in more than 40 % of samples). Fourteen proteins previously described as tumor specific proteins were found in DNPCs of primary BCPs and not one of them was found in HFs (Table 26.2). The data obtained demonstrate the involvement of nuclear, cytosolic and membrane proteins in cfDNA circulation and a high variability of the DNPC protein spectrum between patients. The presence of cancer related proteins only in DNPC from BCPs blood demonstrates the potentiality of DNPC as a source of material for protein-based cancer diagnostics and potential enrichment of cancer related DNA using specific antibodies.

Conflicts of Interest The authors declare no conflict of interest.

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Part IV

Methodology

Digital PCR of Genomic Rearrangements for Monitoring Circulating Tumour DNA

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Abstract

Identifying circulating tumour DNA (ctDNA) for monitoring of cancer therapy is dependent on the development of readily designed, sensitive cancer-specific DNA markers. Genomic rearrangements that are present in the vast majority of cancers provide such markers.

Tumour DNA isolated from two fresh-frozen lung tumours underwent whole genome sequencing. Genomic rearrangements were detected using a new computational algorithm, GRIDSS. Four genomic rearrangements from each tumour were chosen for further study using rearrangement-specific primers. Six of the eight rearrangements tested were identified as

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tumour-specific, the remaining two were present in the germline. ctDNA was quantified using digital PCR for the tumour genomic rearrangements in patient blood. Interestingly, one of the patients had no detectable ctDNA either prior to or post surgery although the rearrangements were readily detectable in the tumour DNA.

This study demonstrates the feasibility of using digital PCR based on genomic rearrangements for the monitoring of minimal residual disease. In addition, whole genome sequencing provided further information enabling therapeutic choices including the identification of a cryptic *EGFR* exon 19 deletion in one patient and the identification of a high somatic mutation load in the other patient. This approach can be used as a model for all cancers with rearranged genomes.

Keywords

Whole genome sequencing • Droplet digital PCR • Lung cancer • Genomic rearrangement • Liquid biopsy

Introduction

Analysis of circulating tumour DNA (ctDNA) is a disruptive new technology enabling relatively non-invasive monitoring of cancer patients. However, ctDNA cannot be distinguished from the abundant normal circulating free DNA without the availability of tumour-specific DNA biomarkers. Most studies have used somatic mutations to identify ctDNA in cancer patients (e.g. Dawson et al. 2013, Newman et al. 2014). The major limitation of the mutation-based approach is that a substantial proportion of tumours do not harbor recurrent somatic mutations for which assays are readily available. Moreover, assays for detection of mutational markers are often difficult to optimise in the context of the high amounts of corresponding normal circulating free DNA.

On the other hand, assays detecting genomic rearrangements are inherently highly sensitive and specific. As genomic rearrangements create new genomic sequences, PCR assays that flank the breakpoint of the rearrangements allow specific amplification of the rearranged templates with exquisite sensitivity and specificity. Somatic genomic rearrangements are present in nearly every tumour and have been demonstrated as excellent tumour-specific markers for monitoring purposes in cancer (Leary et al. 2010, McBride et al. 2010). Whereas patient specific assays do need to be developed, they are relatively easy to design and optimize, and inexpensive to run.

In this study, we utilised two new features to be able to more efficiently use a personal set of genomic rearrangement markers for monitoring and accurately measuring the amount of ctDNA in cancer patients: (i) bioinformatic identification and annotation of genomic rearrangements using a novel algorithm (Cameron et al., submitted) and (ii) digital droplet PCR (ddPCR) of chromosome rearrangements to accurately quantify ctDNA levels in patients' blood to monitor any changes during cancer treatment.

Materials and Methods

Samples

Lung tumours were collected from two stage II lung cancer patients (PL1 and PL2) who underwent surgical resection and received adjuvant chemotherapy at the Austin Hospital, Melbourne, Australia. A preoperative blood sample and post-operative blood samples were collected from the patients. Cell-free plasma was separated from whole blood within 3 h of collection using two centrifugation steps of 800 and 1600 RCF for 10 min each. Buffy coat was also

collected to extract germline DNA. Both plasma and buffy coat samples were stored at −80 °C until DNA extraction. This study was approved by the Institutional Review Board of Human Research Ethics Committee.

DNA Extraction

Genomic DNA was extracted from fresh frozen and matched formalin-fixed tumours and buffy coat using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Plasma DNA was extracted from 2 mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) by eluting twice with 50 μL of ACE buffer.

Identification of Genomic Rearrangements

Fresh frozen tumour DNA was used for paired-end whole genome shotgun sequencing using the Illumina HiSeq X™ Ten platform with a mean 35x sequencing coverage. Paired FASTQ files were aligned against the UCSC hg19 assembly using Bowtie 2 version 2.2.5 and the duplicate reads were marked using Picard MarkDuplicates. Genomic rearrangements were identified using a new computational algorithm, Genomic Rearrangement IDentification Software Suite (GRIDSS) version 0.5.0 (Cameron et al., submitted for publication).

Validation of Genomic Rearrangements

Primers were designed to flank the genomic breakpoints and to generate short amplicons of less than 100 bp for amplification of plasma DNA. The validation experiments were carried out either on a CFX Connect Real-time PCR instrument (Bio-Rad, Hercules, CA) or using the QX200 droplet digital PCR system (Bio-Rad).

For the CFX instrument, the PCR reaction in a final volume of 20 μL contained: 1× PCR buffer, 2.5 mM $MgCl_2$, 100 nM each primer, 200 μM of dNTPs, 5 μM of SYTO 9, 0.5 U of HotStar Taq DNA polymerase (Qiagen) and 10 ng of genomic DNA. The cycling and melting conditions were as follows: one cycle of 95°C for 15 min; 55 cycles of 95°C for 10 s; 68–60°C (gradient PCR) for 20 s and 72 °C for 30 s followed by one cycle of 97°C for 1 min and a melt from 70 to 95°C temperature increasing at 0.2°C per second.

For the QX200 droplet digital PCR system, the PCR reaction was in a final volume of 23 μL, containing 1x EvaGreen ddPCR Supermix, 100 nM each primer and 2 μL of plasma DNA. The manufacturer's instruction was followed for droplet generation, PCR cycling and droplet reading. ddPCR data was analysed using QuantaSoft software (Bio-Rad).

Detection of Genomic Rearrangements in Plasma DNA

The presence of ctDNA with somatic genomic rearrangements was assessed in a pre-operative and a series of post-operative plasma samples using the QX200 droplet digital PCR system. PCR mix was prepared in a final volume of 23 μL, containing 1x EvaGreen ddPCR Supermix, 100 nM each primer and 2 μL of plasma DNA. The manufacturer's instruction was followed for droplet generation, PCR cycling and droplet reading. ddPCR data was analysed using QuantaSoft software.

Detection of EGFR Exon 19 Mutations

EGFR exon 19 mutations were tested by high resolution melting using the conditions previously reported (Clay et al. 2014).

Results

Study Design

The approach in this study consists of two steps, (i) identification and validation of genomic rearrangements by whole genome sequencing of the tumour and (ii) monitoring of the identified genomic rearrangements in the patients' blood.

Identification of Potential Genomic Rearrangements

Whole genome sequencing of two lung tumours was performed using the Illumina HiSeq X™ Ten platform with a mean 35x sequencing coverage. The whole genome sequencing generated an average of 500 million sequence reads. 75 % and 78 % of the reads were successfully mapped to the reference human genome (hg19) for PL1 and PL2 respectively.

GRIDSS was used to identify potential genomic rearrangements that were then ranked in terms of the likelihood of somatic events. GRIDSS performs whole-genome breakpoint assembly, followed by probabilistic structural variant calling based on split read, discordant read pair and breakpoint assembly support. Candidate genomic rearrangements were sorted by breakpoint quality score and the following filters were applied: (1) breakpoints matching to the Database of Genomic Variants sequences within 10 bp, (2) breakpoints with GRIDSS quality score of less than 500, (3) breakpoints on alternate contigs, (4) breakpoints with size less than 1 Mb, (5) breakpoints with 0 supporting split reads, (6) breakpoints with 0 supporting read pairs and (7) breakpoints lacking assembly support from both sides of the breakpoints.

After an initial filtering step, the remaining potential genomic rearrangements were manually curated in descending order of quality score using the following steps: (1) BLAST breakpoint assembly against the NCBI database (all human sequences), (2) variants with a contiguous hit across the putative fusion were marked as germline, (3) inspect breakpoint in Integrative Genomics Viewer (IGV), (4) variants in telomeric and centromeric sequences were excluded, (5) variants forming small translocations were marked as small indel/SVA/LINE insertions based on IGV repeat-masker annotations off the translocated sequence and (6) variants with copy number and BAF change across breakpoint position were marked as putative somatic variants.

Analysis with the GRIDSS algorithm identified 44 and 106 potential genomic rearrangements in PL1 and PL2 lung tumours, respectively (Fig. 27.1). We first searched for the presence of clinically actionable recurrent rearrangements in lung tumours. However, genomic rearrangements involving the *ALK*, *ROS1*, *RET* and *NTRK1* genes were not detected in both tumours.

Curation of the 44 potential genomic rearrangements detected in PL1 further classified those rearrangements into 9 'likely somatic' rearrangements, 12 SINE/VNTR/Alu insertions, eight small intergenic indels (<500 bp), five

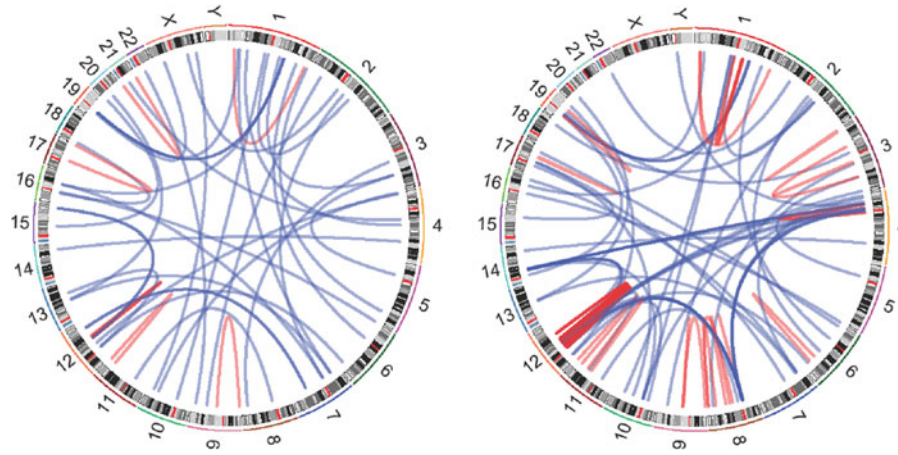

Fig. 27.1 Circos plot of genomic rearrangements detected in the PL1 (*left*) and PL2 (*right*) tumour by GRIDSS. Blue and red lines represent inter- and intra-chromosomal rearrangements respectively

homologies to alternate assembly, three LINE insertions, three events lacking copy number/allele frequency support, two centromeric/telomeric events, and two known germline variants. Among the 106 potential genomic rearrangements detected in PL2, the top 15 potential rearrangements ranked by the GRIDSS algorithm were chosen for further curation. They were classified into nine as 'likely somatic', two as homology to alternate assembly, two as possibly germline, one as a variant in centromere/telomere and one as a LINE insertion.

One interesting finding in the PL2 tumour is that chromosome 12 was involved in about 40% of the genomic rearrangements detected in this tumour, suggestive of chromothripsis (Forment et al. 2012). Among the total 106 potential genomic rearrangements, 44 events were linked with chromosome 12, including 30 intrachromosomal and 14 interchromosomal events.

Validation of Selected Genomic Rearrangements

Among the potential genomic rearrangements identified, the top four markers from each tumour were chosen for validation with the aim of finding several somatic genomic rearrangements for monitoring purposes (Table 27.1). Validated somatic genomic rearrangements were then assessed in both a pre-operative and a series of post-operative blood samples using the droplet digital PCR methodology.

PCR primers were designed to amplify the flanking regions of the eight breakpoints. Genomic DNA extracted from the buffy coat was used as a germline DNA control. Tumour DNA extracted from both fresh frozen and matched formalin-fixed tumours was tested by either qPCR or ddPCR. Genomic rearrangements were called as somatic when only tumour DNAs were amplified and called as germline when both tumour and buffy coat DNA were amplified. Among the four genomic rearrangements tested for PL1, two of the rearrangements were called as somatic and there were two germline events as evidenced by the amplification of the rearrangements from the buffy coat DNA. All of the four rearrangements tested for PL2 were determined to be somatic (Fig. 27.2).

Somatic Variants

Recurrent somatic mutations in the *KRAS*, *BRAF*, *TP53* and *STK11* genes were not detected in either tumour. However, a complex *EGFR* exon 19 deletion mutation involving a deletion of 17 bases and an insertion of 5 bases (p.747_752delinsQH) was detected in the PL1 tumour. The PL1 tumour was previously tested for *EGFR* mutations using the Roche Cobas EGFR Mutation Test kit, but no *EGFR* mutation was detected. To confirm the WGS results, both fresh frozen and matched formalin-fixed tumour DNAs were tested for *EGFR* exon 19 mutations using high resolution melting analysis. The melt-

Table 27.1 Genomic rearrangements selected for the validation study

	5′ end sequence		3′ end sequence			
Sample	Chromosome	Position	Chromosome	Position	Type	Validation
PL1	12	+73141508	12	+77408569	Intrachromosomal	Somatic
PL1	2	+77407530	12	+73140626	Interchromosomal	Somatic
PL1	6	+382460	9	−33428529	Interchromosomal	Germline
PL1	2	+35879356	9	+98459861	Interchromosomal	Germline
PL2	12	+60219721	12	−69680769	Intrachromosomal	Somatic
PL2	12	+71235099	12	+67042226	Intrachromosomal	Somatic
PL2	12	+39812820	12	+59398710	Intrachromosomal	Somatic
PL2	12	+69834954	12	+52749543	Intrachromosomal	Somatic

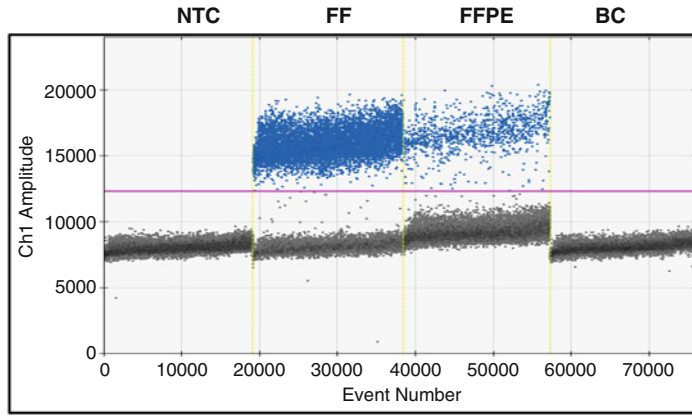

Fig. 27.2 A representative somatic genomic rearrangement detected in PL2 by ddPCR. No Template Control (NTC), Fresh frozen tumour (FF), Formalin-fixed paraffin-embedded tumour (FFPE) and Buffy coat (BC)

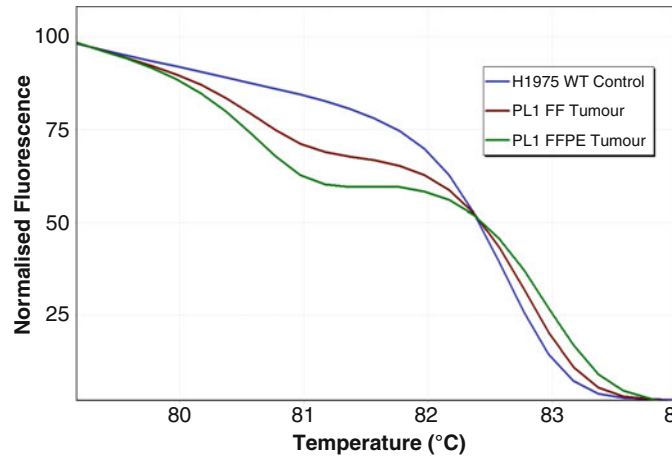

Fig. 27.3 Normalised plot of *EGFR* exon 19 HRM results. The melting profiles of both PL1 fresh frozen and formalin-fixed tumour DNAs were different from that of the wild-type control, H1975

ing profiles of both samples clearly deviated from that of the wild-type control, indicative of the presence of an *EGFR* exon 19 mutation (Fig. 27.3).

Monitoring of Genomic Rearrangements in Plasma

A series of plasma samples were collected from patients PL1 and PL2, at the baseline, 2–3 days after surgery and at regular check-ups. The presence of ctDNA was monitored in the plasma samples using the confirmed somatic genomic rearrangements as ctDNA markers using the droplet digital PCR methodology.

For PL1, two validated somatic genomic rearrangements were tested in three plasma samples collected at the baseline, 2 days and 3 months after surgery. However, ctDNA containing those genomic rearrangements was not detected in any of the plasma samples including the pre- and post-operative plasma samples.

For PL2, four somatic genomic rearrangements were tested in plasmas collected at the baseline, 3 days, 3 months and 5.5 months after surgery. ctDNA containing the four different genomic rearrangements was clearly detectable at the baseline plasma, although the level of each ctDNA marker was quite different (Fig. 27.4). The most abundant ctDNA marker was estimated

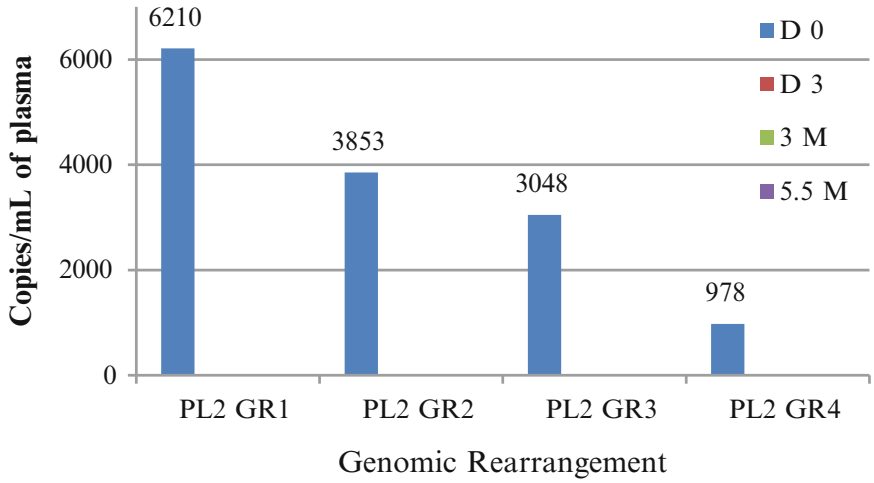

Fig. 27.4 Quantification of ctDNA in plasma samples using four different rearrangement markers in PL2 using droplet digital PCR

to be present at 6210 copies/mL of plasma and the least abundant rearrangement ctDNA marker was estimated to be present at 978 copies/mL of plasma. There was more than a sixfold difference in terms of number of ctDNA between the most and least abundant markers. A similar relative abundance was seen in the tumour DNA when assessed by ddPCR. Those rearrangement ctDNA markers, however, were no longer detectable in three post-operative plasmas collected after surgery (3 days, 3 months and 5.5 months), indicating a dramatic reduction of the ctDNA in blood after surgery.

To rule out the possibility of the absence of amplifiable plasma DNA, all plasma DNA samples were tested using an independent ddPCR assay for the *BRAF* exon 15 wild-type sequence (Tsao et al. 2015). All plasma DNA samples contained amplifiable *BRAF* templates, indicating the presence of amplifiable plasma DNA in all plasma samples (data not shown).

Discussion

The approach used in this study is based on the personalized analysis of rearranged ends (PARE) approach (Leary et al. 2010, McBride et al. 2010). Nearly every tumour harbors a 'personal' set of genomic rearrangements and such personal rearrangements were used as clonal markers of solid tumour cells. An advantage of the PARE approach relies on the nature of genomic rearrangements. We have implemented an improved bioinformatic analysis and a readily performed droplet digital PCR assay to expedite the clinical translation of this approach.

Digital PCR is an ideal molecular technique for absolute quantification of circulating tumour DNA (ctDNA) in patient's blood. The level of ctDNA in patient's blood has been shown to be a surrogate marker of tumour burden (Diehl et al. 2008; reviewed in Diaz and Bardelli 2014), enabling longitudinal monitoring of tumour response to cancer therapy and detection of minimal residual disease. As circulating cell free DNA from apoptotic normal cells is usually more abundant than ctDNA, detection of ctDNA in patient's blood requires highly sensitive and specific approaches.

Here, we have confirmed that genomic rearrangements represent excellent tumour-specific alterations for cancer patients whose tumours do not harbor recurrent somatic mutations. An attractive advantage of using genomic rearrangements as tumour markers is the ready design of assays that enable detection with inherently high sensitivity and specificity. As somatic genomic

rearrangements create new genomic sequences, detection of genomic rearrangements is achieved by designing the primers targeting the sequences flanking the breakpoints. In addition, other advantages of testing genomic rearrangements include fast turn-around time, potential to detect clinically actionable rearrangements and the low cost of monitoring tests using the rearrangement-specific PCR assays.

Furthermore, considerable somatic mutation information can also be obtained from WGS data. In our study, a rare *EGFR* exon 19 deletion mutation that was not detected by the Roche Cobas EGFR Mutation Test was identified in the PL1 tumour, opening another therapeutic option for this patient. In PL2, a high exonic mutation rate was identified (results not shown) indicating that the patient might respond well to treatment with immune checkpoint inhibitors.

The limitation of the ctDNA-based monitoring approach is that ctDNA is not detectable in every cancer patient's blood and the detection rate differs depending on the tumour type (Bettegowda et al. 2014). Bettegowda et al. reported that ctDNA was detected in all of the bladder and colorectal cancer patients tested, but was detectable in only 10 % of glioma and 30 % of thyroid cancer patients. In lung cancer, ctDNA has been detected in about 50–100 % of patients (Taniguchi et al. 2011, Newman et al. 2014), depending on tumour stage and the detection method used, indicating that ctDNA markers can be absent from a patient's blood as seen in our PL1 patient. The tumour of patient PL1 harboured both somatic genomic rearrangements and an *EGFR* exon 19 deletion mutation, but those tumour-specific markers were not detected in all plasma samples. Therefore, the lack of ctDNA markers in a patient's blood does not entirely exclude the possibility of residual tumours in cancer patients.

A disadvantage of this approach is the need to identify rearrangements by relatively expensive whole genome sequencing. However, the disadvantage of the initial cost for whole genome sequencing and bioinformatics is more than compensated by the low cost and high sensitivity and specificity of the assays that can be designed as a consequence of the genomic information as well as the likelihood of identifying other information that may be used to guide the personalised treatment of the patient.

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Conflict of Interest We have no conflict of interest to declare.

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mFast-SeqS as a Monitoring and Pre-screening Tool for Tumor-Specific Aneuploidy in Plasma DNA

28

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Abstract

Recent progress in the analysis of cell-free DNA fragments (cell-free circulating tumor DNA, ctDNA) now allows monitoring of tumor genomes by non-invasive means. However, previous studies with plasma DNA from patients with cancer demonstrated highly variable allele frequencies of ctDNA. Comprehensive genome-wide analysis of tumor genomes is greatly facilitated when plasma DNA has increased amounts of ctDNA. In order to develop a fast and cost-effective pre-screening method for the identification of plasma samples suitable for further extensive qualitative analysis, we adapted the recently described FAST-SeqS method. We show that our modified FAST-SeqS method (mFAST-SeqS) can be used as a pre-screening tool for an estimation of the ctDNA percentage. Moreover, since the genome-wide mFAST-SeqS z-scores correlate with the actual tumor content in plasma samples, changes in ctDNA levels associated with response to treatment can be easily monitored without prior knowledge of the genetic composition of tumor samples.

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Keywords

Liquid biopsy • mFAST-SeqS • Circulating tumor DNA (ctDNA) • Cancer • Plasma-Seq • Copy number alterations (CNA)

Introduction

Development and access to accurate and sensitive screening methods for the early and fast detection of tumor-specific aberrations and identification of therapy-related changes is of utmost importance for optimal therapy management and patient care. Recent progress in the analysis of cell-free DNA fragments (cell-free circulating tumor DNA, ctDNA) now allows monitoring of tumor genomes by non-invasive means (Bettegowda et al. 2014; Diaz and Bardelli 2014; Heitzer et al. 2013a, b, c, d; Lianidou et al. 2014; Lim et al. 2014). One possibility to make use of a ctDNA is a genome-wide analysis without knowledge of any specific changes that are present in the primary tumor, which is important, in particular, either for tumor entities that lack recurrent genetic changes or patients where no tumor material is available. Most genome-wide methods currently lack sensitivity and the amount of tumor-specific DNA has to be above 5–10 % (Chan et al. 2013a; Forshew et al. 2012; Heitzer et al. 2013d; Murtaza et al. 2013). However, the levels of ctDNA vary dramatically, ranging from less than one to more than 90 % (Bettegowda et al. 2014; Heitzer et al. 2013b; Leary et al. 2012; Thierry et al. 2014). Therefore, the availability of pre-screening methods to assess the amount of tumor DNA and to select samples that are suitable for an extensive qualitative genome-wide analysis would be highly beneficial in order to save costs and time.

To this end, we modified the Fast Aneuploidy Screening Test-Sequencing System described by (Kinde et al. 2012), which is a 2-step PCR amplifying eight subfamilies of long interspersed nucleotide element-1 (L1) retrotransposons that are uniquely mappable across the genome. We used the method to pre-screen plasma DNA samples in order to evaluate whether or not the fraction of tumor-specific DNA is sufficient for our genome-wide plasma-Seq analysis (Heitzer et al. 2013d). While Kinde at al. performed read count and z-score analysis for whole chromosomes (Kinde et al. 2012), we expanded the analysis to the chromosome arms.

Methods

Patient Samples and Cancer Cell Lines

A set of 24 female controls and 18 male controls without malignant disease were collected at the Departments of Urology, Gynecology, Cardiology and Endocrinology at the Medical University of Graz. Blood samples from breast (n = 28) and prostate cancer patients (n = 61) were obtained from either the Departments of Urology and Gynecology or the Division of Clinical Oncology, Department of Internal Medicine at the Medical University of Graz. In order to validate reproducibility of the assay, cancer cell lines HT29 and MCF7 were included (ATCC, Manassas, VA, USA).

Extraction of Genomic DNA and Plasma DNA

High molecular weight genomic DNA from cultured cell lines was extracted using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Plasma DNA was prepared using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as previously described (Heitzer et al. 2013b).

mFAST-SeqS Assay

The method was described in detail previously (Belic et al. 2015). Briefly, 20 ng of cell line DNA and 0.1–5 ng of plasma DNA were amplified with Phusion Hot Start II Polymerase in five PCR cycles using target-specific L1 primers. PCR products were purified using AMPure Beads (Beckman Coulter, Brea, CA, USA) and resuspended in 12 µl 1×TE-buffer. 10 µl of purified PCR products were directly used for the second PCR (18 cycles for plasma DNA, 15 cycles for all other samples) in which Illumina specific adaptors and indices were added (for primer sequences please see (Belic et al. 2015)). PCR products were quality checked and quantified on an Agilent Bioanalyzer using a 7500 DNA kit (Agilent, Santa Clara, USA). L1 amplicon libraries were pooled equimolarly and sequenced on an Illumina MiSeq generating 150 bp single reads. Sequence reads were aligned to the hg19 genome using bwa (version 0.7.4). For each chromosome arm, reads having a mapping quality >15 were counted using an in-house script. In order to correct for different sequencing yields, read counts were normalized with the total read count per sample. We applied a z-score statistic to test for over- and under-representation of each chromosome arm. Hence, we calculated mean and standard deviation of normalized read-counts for those controls, of which a minimum of 100.000 reads were obtained after mapping quality filter step (n = 17 for male, n = 19 for female). In order to get a general overview of aneuploidy in our samples, we introduced a genome-wide z-score. Therefore, normalized read counts per chromosome arm were squared and summed. We again applied z-score statistics for the summation of the normalized read-counts by calculating mean and standard deviations for the respective controls.

Plasma-Seq

The method was described in detail previously (Heitzer et al. 2013d). Briefly, shotgun libraries were prepared using the TruSeq DNA LT Sample preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. For plasma samples, we slightly changed the protocol as follows: we used 5–10 ng of input DNA, we omitted the fragmentation step and for selective amplification of the library fragments that have adapter molecules on both ends, we used 20–25 PCR cycles. The libraries were sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA).

Results

Validation of mFAST-SeqS with DNA from Cancer Cell Lines

First, we validated the reliability and reproducibility of the mFAST-SeqS assay by analyzing different dilutions of HT29 DNA in a background of normal DNA (100 %, 50 %, 10 % 5 %, 0 %). We obtained good reliability and repeatability of the genome-wide z-score with only small inter-assay ($r = 0.998$) and intra-assay ($r = 0.992$) variations. Linear regression analysis revealed a good correlation between genome-wide mFAST-SeqS z-scores and the dilution of HT29 cell lines (mean $r = 0.974$). When we evaluated our mFAST-SeqS assay by analyzing cancer cell lines MCF7 and HT29 with our plasma-Seq approach, copy number alterations (CNAs) were highly concordant. High level amplifications of specific parts of chromosomes resulted in a high overall chromosome-arm specific z-score. For instance, the high level amplifications of chromosome 8q were reflected with 8q-specfic z-scores of 42.2 and 15.0, respectively (Fig. 28.1). Loss of the short arm of chromosome 3 in HT29 with a complete loss of the region close to the centromere resulted in a 3p-specific z-score of −20.1 (Fig. 28.1). For some chromosomes, the chromosome-arm specific z-scores were below the threshold of five although circulating nucleic acids (CNAs) were detected with plasma-Seq. This can be explained by the co-occurrence of gains and losses at the same chromosome arm resulting in a balanced copy number status as exemplified for chromosome 1 of MCF7 (Fig. 28.1).

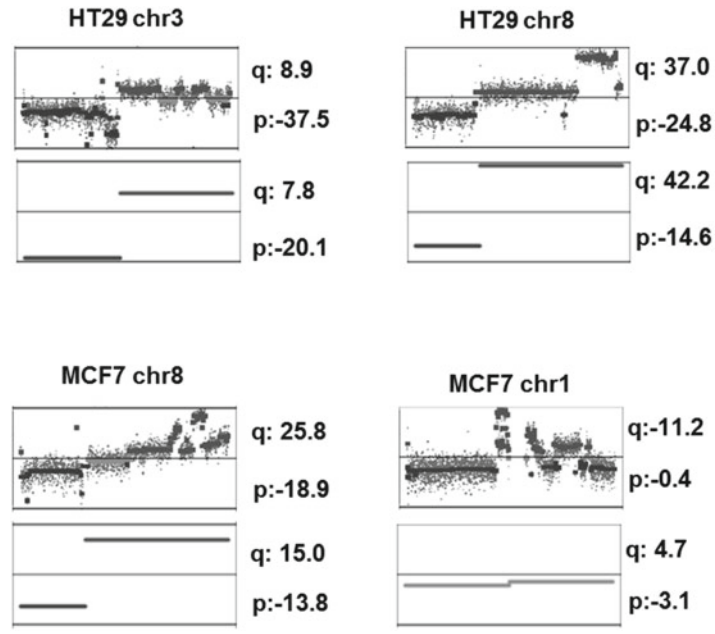

Fig. 28.1 Comparison of mFAST-SeqS profiles and copy number profiles established with plasma-Seq. Selection of particular chromosomes with *high* or *low* chromosome-specific z-score reflecting high level amplifications or loss of chromosomal material in different cell lines. Co-occurrence of gains and losses at the same chromosome arm results in a balanced chromosome specific copy number status established with mFAST-SeqS

mFAST-SeqS for Minimal-Invasive Aneuploidy-Screening in Cancer Patients

In order to evaluate whether this assay can indeed be used as a pre-screening tool for the presence of higher levels of tumor-specific DNA in plasma, we analyzed a total of 89 cancer patients. As a training set, 28 plasma samples from patients with metastatic breast cancer were used. In all samples with a genome-wide mFAST-SeqS above five CNAs could be observed with plasma-Seq, whereas samples with a genome-wide z-score <5 showed balanced plasma-Seq profiles (Fig. 28.2). To validate these results we analyzed a set of 61 prostate cancer patients with various disease stages. Of those, 25 (41.0 %) samples had a genome-wide z-score below five. Not surprisingly, these patients included all patients under surveillance and nine of ten patients with localized cancer after prostectomy, of which a low fraction of tumor-specific DNA was expected. However, 12 metastasized patients also had a z-score below five indicating that the amount of tumor-specific DNA was quite low in these patients. Again, after plasma-Seq, CNAs could be only be observed in samples with a genome-wide z-score higher than five indicating that a high genome-wide z-score reflected a high amount of tumor-specific CNAs and therefore also a high fraction of tumor-specific DNA in plasma. In order to investigate whether the genome-wide z-score is indeed correlated with ctDNA levels, we compared allele frequencies (mAFs) of mutations in driver genes identified by targeted re-sequencing with the mFAST-SeqS z-score and observed a strong correlation. On the basis of linear regression, a z-score of five would predict a mAF of 10.5 %.

mFAST-SeqS as a Monitoring Tool for Changing Levels of Tumor DNA in Plasma

Since the genome-wide z-score correlated with levels of tumor DNA in the circulation, we investigated whether the z-score can also be used for monitoring purposes. Thus, we evaluated the utility of the genome-wide z-score as a monitoring tool for treatment response by analyzing samples from prostate cancer patients before and during ADT. As expected, the genome-wide z-score decreased massively in patients who responded

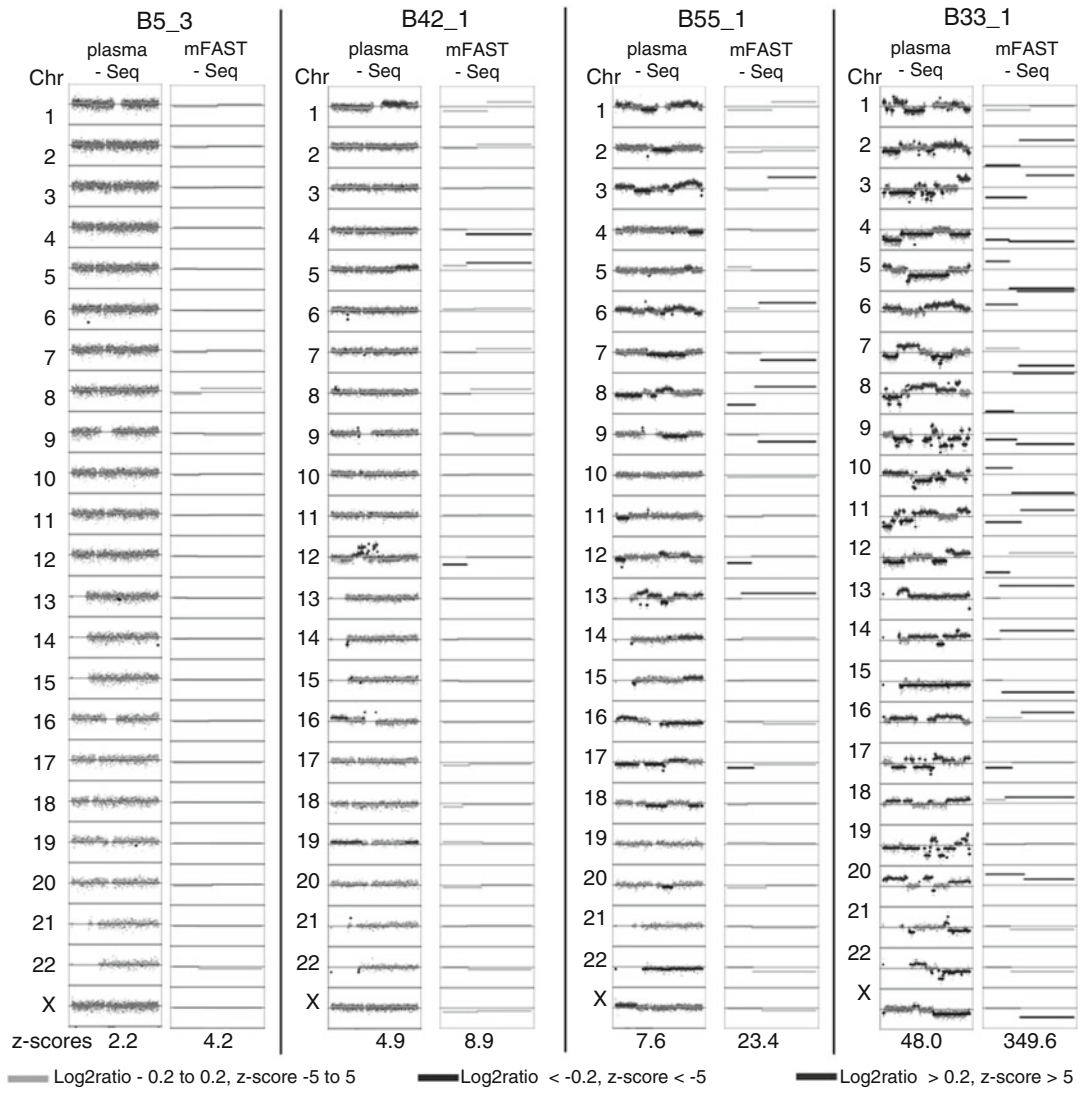

Fig. 28.2 mFAST-SeqS assay of plasma samples from patients with metastatic breast cancer. Comparison of mFAST-SeqS profiles (*right panels*) and copy number profiles established with plasma-Seq (*left panels*) of selected samples. Samples with genome-wide z-score above five show copy number alterations established with plasma-Seq

well to the therapy (Fig. 28.3a, b). Consistent with mFAST-SeqS, the mutant allele frequencies of mutations identified with targeted re-sequencing were no longer detectable with deep amplicon sequencing after 2 months of treatment (Fig. 28.3a, b). In addition, plasma-Seq resulted in a balanced copy number profile despite a variety of CNAs in the first samples, also confirming the reduction of tumor DNA (Fig. 28.3a, b). In contrast, in patients who did not respond to ADT treatment, genome-wide z-scores neither changed nor even increased and the same applied to mutant allele frequencies and CNAs (Fig. 28.3c).

For one patient we received three samples that allowed us to monitor changing clonal patterns. When we obtained the first blood sample the patient was already resistant to androgen deprivation therapy (ADT) because of a high level amplification of the androgen receptor gene (AR) located at the X chromosome (Fig. 28.4). mFAST-

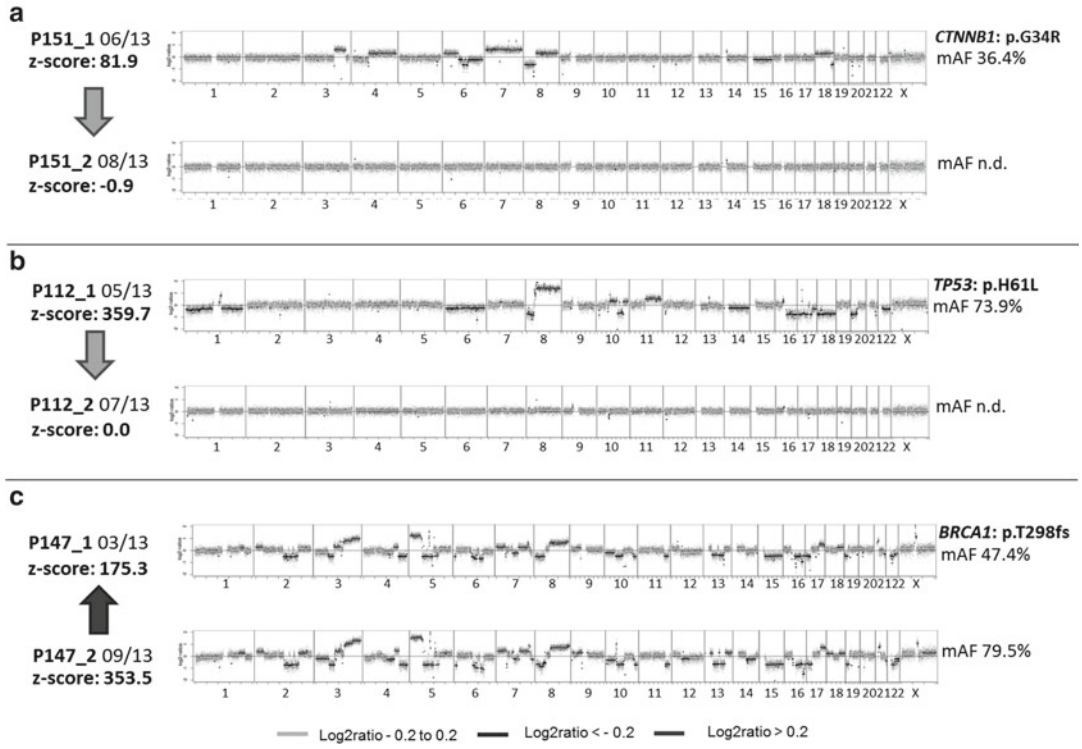

Fig. 28.3 Monitoring of patients with metastatic prostate cancer under androgen deprivation therapy (ADT) (**a, b**) Genome-wide mFAST-SeqS z-score decreased in patients who responded well to ADT therapy. Consistent with mFAST-SeqS z-scores, the mutant allele frequencies (mAF) of mutations identified with targeted re-sequencing were no longer detectable with deep amplicon sequencing after 2 months of treatment. (**c**) A patient who did not respond to ADT treatment is shown. Genome-wide Z-scores increased after 6 months of treatment and the same applied to mutant allele frequencies and CNAs

SeqS revealed a very high genome-wide z-score of 300. After switching to chemotherapy, the patient responded quite well; this was reflected in decreased tumor DNA levels and a drop in the mFAST-SeqS z-score to six. However, after several months the patient progressed again and the level of ctDNA increased as shown in the mFAST-SeqS z-score. In addition, using plasma-Seq we observed the emergence of novel CNAs, e.g. on chromosomes 2 and 4, while other changes disappeared upon *AR* amplification. Moreover, we were able to identify two somatic mutations in this patient, one in *EP300* and another one in *TP53*. Both mutations dropped after the switch to chemotherapy, but at the time of progression, only the *TP53* increased while the *EP300* mutation was no longer detectable further confirming the clonal switch of this tumor. If we had followed the *EP300* mutation alone we would have missed the massive increase in tumor DNA associated with progression in this patient (Fig. 28.4).

Discussion

In this study, we describe an extended application of the previously established FAST-SeqS method (Kinde et al. 2012) for fast and cost-effective pre-screening of tumor-specific aneuploidy in plasma samples. We show that mFAST-SeqS serves as a valuable pre-screening tool to identify samples where tumor specific changes are most likely identified with less sensitive methods such as low-coverage whole genome sequencing or

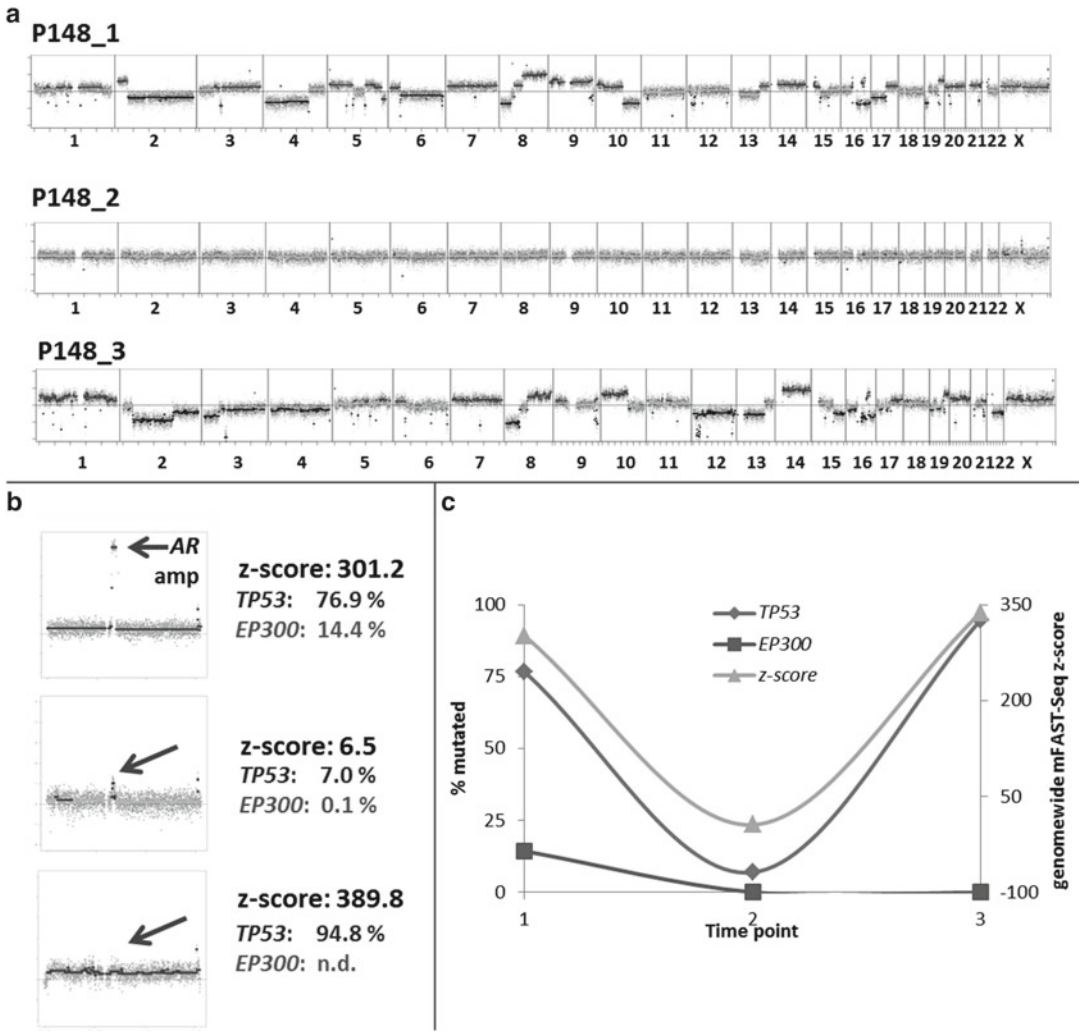

Fig. 28.4 Monitoring of changing clonal patterns. The patient was already resistant to ADT when the first blood sample was obtained. (**a**) Changing copy number pattern arose after initial response to chemotherapy. (**b**, **c**) mFAST-SeqS revealed a very high genome-wide z-score of 300. Initial response to chemotherapy was reflected in a decrease of mFAST-SeqS z-score and mutant allele frequencies of *TP53* and *EP300* mutations. At the time of progression, mFAST-SeqS and *TP53* mutation increased while the *EP300* mutation was no longer detectable indicating a clonal switch

exome sequencing. Since genome-wide or exome-wide analysis of ctDNA can lead to high costs, a pre-screening using the mFAST-SeqS assay can help to reduce costs as samples with lower tumor can be identified at an early stage and can be assessed whether the samples is worth analyzing with genome-wide methods or is rather referred to more sensitive approaches (Fig. 28.5).

One of the key challenges in the analysis of ctDNA is the fact that the fraction of circulating DNA that is derived from the tumor can range between 0.01 % to more than 90 % (Bettegowda et al. 2014; Heitzer et al. 2013b; Leary et al. 2012; Thierry et al. 2014). Although there exist many promising genome-wide approaches for the analysis of ctDNA (Chan et al. 2013a, b; Heitzer et al. 2013b, d; Leary et al. 2012), most of these methods lack sensitivity and may not be used if the fraction of umor-specific DNA is below a specific threshold of 5–10 %. With our

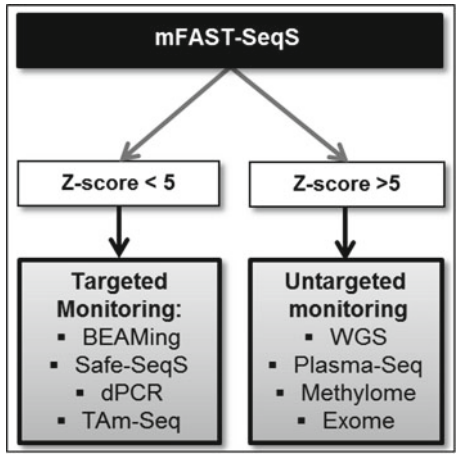

Fig. 28.5 mFAST-Seq as a decision support tool in order to select the most appropriate analysis strategy
If the mFAST-SeqS z-score is below five, targeted approaches should be used for ctDNA analysis that can detect mutant alleles as low as 0.01–0.5 %. In cases with mFAST-SeqS z-scores above five, untargeted approaches can be used which require a higher fraction of ctDNA

established plasma-Seq method tumor specific CNAs can be detected in the circulation at levels ≥10 % of circulating tumor DNA with a sensitivity of >80 % and specificity of >80 % (Heitzer et al. 2013d). Using this approach we analyzed a set of colorectal cancer patients under anti-EGFR therapy and cohort of metastasized breast cancer patients. While tumor-specific changes were identified in all CRC patients (Mohan et al. 2014), only 46 % of the breast cancer patients showed CNAs with our plasma-Seq method (Heidary et al. 2014) indicating that for a subset of samples more sensitive methods are required to identify tumor-specific changes in the circulation. Using mFAST-SeqS we are now able to roughly estimate the amount of tumor-specific DNA in plasma without any knowledge of specific aberrations of the primary tumor. In those cases with a genome-wide z-score above five tumor-specific CNAs can be established with plasma-Seq (Fig. 28.5). Furthermore, the obtained results revealed highly concordant copy number profiles of mFAST-SeqS and plasma-Seq indicating that mFAST-Seq can additionally give insights into CNAs at a chromosome arm level.

Since genome-wide z-scores reflect the level of ctDNA in the circulation, mFAST-SeqS might also be a suitable monitoring tool for treatment response. Monitoring of specific mutations might miss clonal changes in the tumor genome occurring during tumor progression or under the selective pressure of therapies as exemplified in Fig. 28.4. mFAST-SeqS is able to reflect changes in ctDNA levels associated with treatment response and progression independently of a prior knowledge of the characteristics of the primary tumor.

Altogether, mFAST-SeqS is a not only a cost-effective screening tool for the evaluation of the fraction of tumor specific DNA, but also an untargeted strategy for the assessment of changes in ctDNA levels as a response to certain therapies.

Conflict of Interest None.

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Methodological Variables in the Analysis of Cell-Free DNA

29

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Abstract

In recent years, cell-free DNA (cfDNA) analysis has received increasing amounts of attention as a potential non-invasive screening tool for the early detection of genetic aberrations and a wide variety of diseases, especially cancer. However, except for some prenatal tests and BEAMing, a technique used to detect mutations in various genes of cancer patients, cfDNA analysis is not yet routinely applied in clinical practice. Although some confusing biological factors inherent to the *in vivo* setting play a key part, it is becoming increasingly clear that this struggle is mainly due to the lack of an analytical consensus, especially as regards quantitative analyses of cfDNA. In order to use quantitative analysis of cfDNA with confidence, process optimization and standardization are crucial. In this work we aim to elucidate the most confounding variables of each preanalytical step that must be considered for process optimization and equivalence of procedures.

Keywords

Cell-free DNA • Cancer • Preanalytical factors • Prenatal diagnostics • Quantitative analysis

Introduction

Since the discovery of cfDNA in human blood, the focal point of most cfDNA studies has been to scrutinize it as a potential non-invasive diagnostic and prognostic marker for solid tumours. Although considerable progress has been made in this regard, it is as yet a partial victory. Except for some prenatal tests (Chiu et al. 2013) and BEAMing (Lawrie et al. 2008), cfDNA analysis

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has not really been translated to clinical practice and routine application seems distant. This can be ascribed to three factors that overlap: (i) a lack of knowledge regarding the origin and function of cfDNA, (ii) insufficient molecular characterization, and (iii) the absence of an analytical consensus. Although it is generally accepted that the last mentioned is a major obstacle, very few have addressed it (El Messaoudi et al. 2013). As a result, most research groups have, by their own discretion, developed in-house procedures that are prejudicial to the smooth translation of cfDNA analysis to clinical practice (El Messaoudi and Thierry 2015). In a recent review we have addressed this issue, and focussed specifically on quantitative analysis of cfDNA (Bronkhorst et al. 2015). In this, we have reached two prominent conclusions. Firstly, the lack of head-to-head comparative studies between the different techniques utilized has led to conflicting results between research groups. Secondly, although it is clear from the literature that a single quantitative assessment is of very limited value, it is discernible that an analysis of the kinetics of cfDNA concentration will be a strong auxiliary component to qualitative characterization. However, in order to confidently use quantitative analysis for this purpose a major proviso becomes crucial, i.e., the optimization and standardization of procedures (van der Vaart and Pretorius 2010).

Therefore, in this work we have aimed to elucidate the most confounding variables at each preanalytical step that need to be considered in this endeavour. Using the growth medium of cultured cells as a source of cfDNA, we assessed the influence of variations to the centrifugation regime, storage temperature, thawing temperature, and storage tube type on the yield of cfDNA. Moreover, regarding the isolation of cfDNA, we assessed the effect of tube type, type of denaturing agent, binding buffer, elution buffer volume, elution regime, and the tube in which isolated cfDNA is stored. Although growth medium was used instead of blood, there are many points of contact between the methods used. The results will thus be relevant to both *in vitro* and *in vivo* studies.

Materials and Methods

Below is described the standard method we used for quantitative analysis of cfDNA. The details of the changes to this basic protocol is described in the results and discussion, but more detailed descriptions of these changes can be found in Bronkhorst et al. (2015).

Cell Culturing

Culture medium of the human bone cancer (osteosarcoma) cell line 143B (ATCC® CRL-8303TM) was used as a source of cfDNA. Cells were cultured in T75 flasks in Dulbecco's Modified Eagle's medium (DMEM) (HyClone; #SH30243.01) fortified with 10 % fetal bovine serum (Biochrom; #S0615) and 1 % penicillin/streptomycin (Lonza; #DE17-602E) at 37 °C in humidified air and 5 % CO_2.

Extraction of cfDNA

CfDNA was extracted with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany; #1502/001), according to the instructions described by the PCR clean-up user manual. For each biological replicate, cfDNA was extracted in triplicate. For every sample, 600 µL of growth medium was mixed with 1200 µL of binding buffer. Samples were then vortexed, the entire volume of growth media was added to the spin column in small regiments, and centrifuged

at 11,000 g for 1 min at room temperature. The columns were then washed twice, followed by the elution of cfDNA into 20 μL of elution buffer.

Quantification of Cell-Free DNA

PCR amplification of cfDNA was measured using a real-time quantitative assay for the β-globin gene. All assays were performed on a Rotor-Gene Q detection system (Qiagen) using a 72 well ring-setup. The reaction mixture consisted of 2 μL DNA and 23 μL master mix, which was composed of 8.1 μL H_2O, 12.5 μL TaqMan Universal MasterMix (Life technologies; #1502032), 0.4 μL of 10 μM dual fluorescent probe 5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3′, and 1 μL of 10 μM forward and reverse primers, respectively. The primers used were: F1, 5′-GTG CAC CTG ACT CCT GAG GAG A-3′, and R1, 5′-CCT TGA TAC CAA CCT GCC CAG-3′. These primers and the probe were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific). PCR conditions were set to: 95 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C, 1 min annealing at 60 °C, followed by 30 s extension at 72 °C.

Results and Discussion

Using the literature as a guideline, several changes to the standard cfDNA handling protocol were investigated. The details of these modifications are described in (Bronkhorst et al. 2015). Results are summarized in Figs. 29.1, 29.2, 29.3 and 29.4 and are discussed in the figure captions.

Conclusion

By conducting a literature survey, we have previously identified and discussed several methodological variables that could potentially influence quantitative measurements of cfDNA (Bronkhorst et al. 2015). From this, we have selected the most confounding variables to evaluate further experimentally. Using the growth medium of cultured cells as a source of cfDNA, we found variations to the centrifugation regime, storage temperature, thawing temperature, and storage tube type to affect the yield of cfDNA considerably. Furthermore, regarding the isolation of cfDNA, we found variations to the type of denaturing agent, binding buffer, elution buffer volume, elution regime, and the tube in which isolated cfDNA is stored to also greatly affect yield. Indeed, some changes resulted in a significant increase of cfDNA yield. A combination of certain changes should appease cfDNA extraction which may appease quantification, gene expression profiling and sequencing. However, it is evident that too many changes work against each other, and establishing the optimal combination of changes requires further experimentation. In conclusion, we propose that many of these variations have not yet been considered, and that it should be useful to consider them when optimizing protocols and setting up a standard operating procedure.

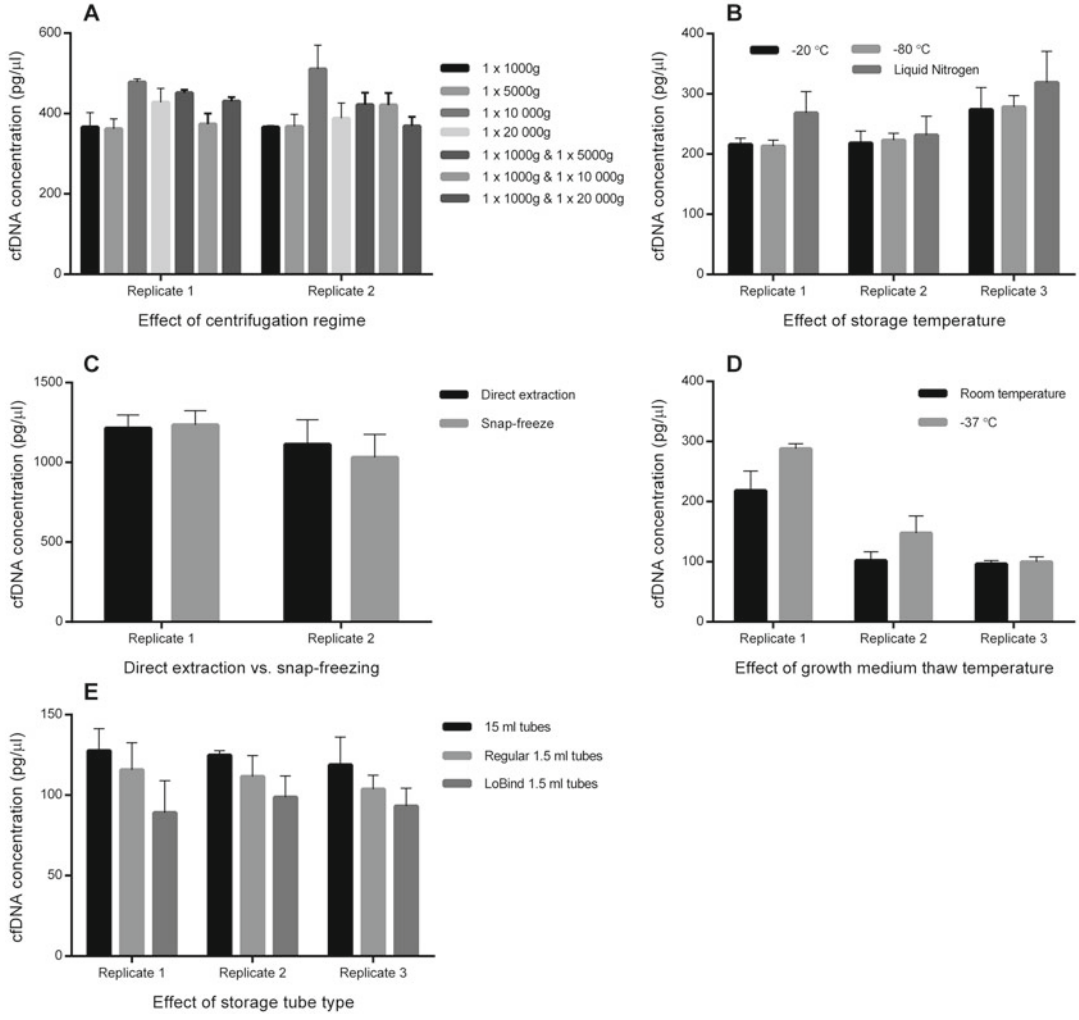

Fig. 29.1 The effect of sample handling on quantitative measurements of cfDNA. (**a**). The effect of varying centrifugal forces and repetition. Samples were centrifuged for 10 min at the different forces. In both experiments, one centrifugation at 10,000 g delivered the highest yield. (**b**). Changes in cfDNA yield during storage. After the medium was centrifuged, it was transferred to fresh tubes and stored until cfDNA was extracted. In all three experiments, storage at −20 °C and −80 °C delivered comparable results, while snap-freezing the samples in liquid nitrogen before storage at −80 °C resulted in an increase of cfDNA yield, although only replicate one is statistically significant. It could be argued that this extra step dissociates the DNA from the protein complexes with which it is associated. However, in (**c**) (results taken from Fig. 29.2b.) we see that the amount of cfDNA extracted after snap-freezing correlates with the amount of cfDNA that is extracted from medium directly after collection. It can thus be argued that in the case of storage at −20 °C and −80 °C a fraction of cfDNA is lost. (**d**). The effect of the medium thawing temperature on the amount of cfDNA extracted. In all experiments, the cfDNA yield was higher when the medium was thawed at 37 °C as opposed to room temperature. This is convenient, given that thawing at 37 °C takes only about 3 min, whereas thawing at room temperature takes at least 10 times longer. (**e**). The loss of cfDNA due to tube type. Storage of medium in three different tubes resulted in three different amounts of cfDNA extracted. Storage in nuclease-free 15 mL tubes resulted in the least amount of DNA loss, followed by regular 1.5 mL Eppendorf tubes, with DNA LoBind Eppendorf tubes resulting in the most loss of cfDNA. In the case of the 15 mL tubes, it may be argued that less cfDNA is lost due to a smaller tube area-to-sample ratio than the 1.5 mL tubes

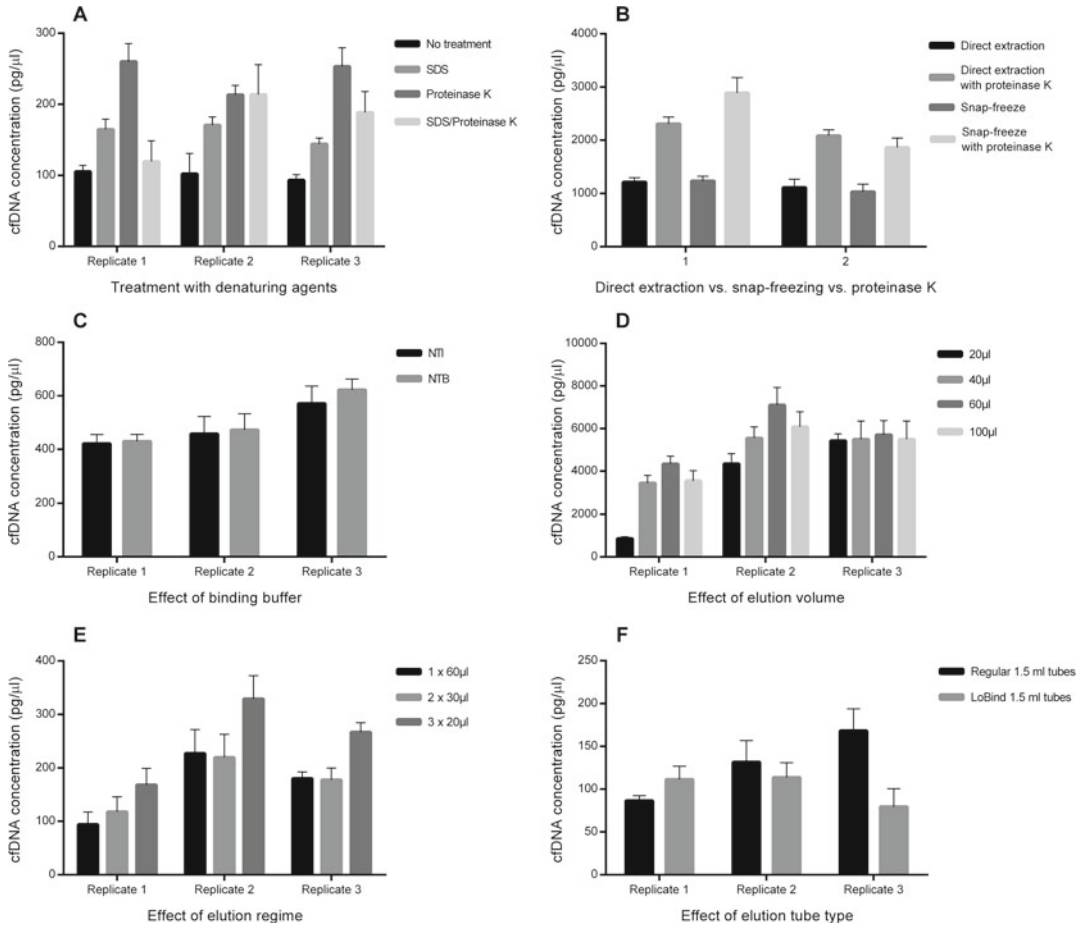

Fig. 29.2 The effect of modifications to the extraction protocol on quantitative measurements of cfDNA. (**a**). The effect of the addition of denaturing agents. The use of SDS increased the yield of cfDNA by approximately 50 % while proteinase K increased the yield by more than 100 %, whereas a combination of the two delivered confusing results. (**b**). A comparison of cfDNA yield between direct extraction and snap-freezing, with and without the use of proteinase K. Since the medium of all the samples in the previous experiment was snap-frozen, this experiment was performed to determine whether the high yields of cfDNA are wholly or only partly due to the addition of proteinase K and not partly due to snap-freezing. It is clear that snap-freezing does not increase the amount of cfDNA extracted and that the high yields can be ascribed solely to the addition of proteinase K. (**c**). The effect of binding buffer type. In the case of buffer NTB, the ratio of sample to buffer is 1:5. In the case of extractions where buffer NTI is used, the sample to buffer ratio is only 1:2. Furthermore, we wanted to compare these two buffers in the absence of SDS. The use of buffer NTB resulted in only a very slightly higher yield of cfDNA than that obtained with buffer NTI. Since the difference is not statistically significant and since the use of NTB is more arduous, this convention is not advised. (**d**). The effect of elution volume. CfDNA was extracted and eluted into 20 µL, 40 µL, 60 µL and 100 µL of elution buffer, respectively. Except for replicate three where no statistically significant difference was observed, DNA yield increased as the elution volume increased up to 60 µL where it reached its peak, and declined rather dramatically after that. (**e**). The effect of elution regime. CfDNA was extracted and eluted into 20 µL of elution buffer and repeated twice more to have a final volume of 60 µL. This was followed by the elution of DNA into 30 µl of elution buffer and repeated once more to achieve a final volume of 60 µL. The former was compared to DNA elution directly into 60 µl of elution buffer. In each experiment, the yield of DNA was significantly higher when eluted in three steps of 20 µL at a time. (**f**). The effect of tube type on the loss of extracted cfDNA. To examine the loss of eluted DNA due to adsorption to the tube walls, we compared regular 1.5 mL Eppendorf tubes with 1.5 mL Eppendorf DNA LoBind tubes. In two of the experiments, the concentration of cfDNA stored in the LoBind tubes was much lower than that of DNA stored in standard tubes. These results agree with the results obtained in Fig. 29.2e

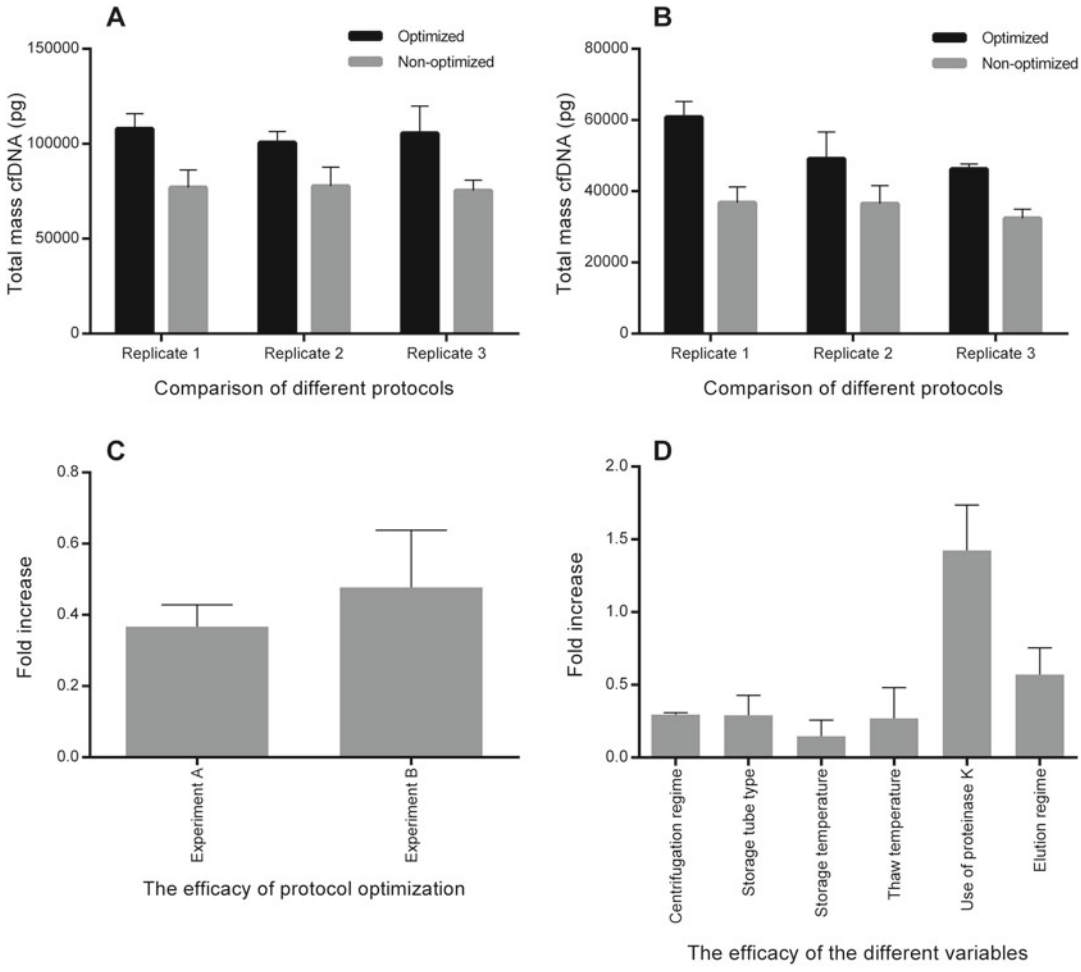

Fig. 29.3 Comparing different extraction protocols. (**a**). The variables that produced the highest increase in cfDNA yield were incorporated into the extraction protocol and compared to a protocol consisting of its less effective counterparts. In the optimized protocol, media were collected and centrifuged at 10,000 g and transferred to fresh 15 mL tubes. The media were then snap-frozen in liquid nitrogen and stored at −80 °C. The samples were then thawed at 37 °C and incubated with proteinase K (1.5 mg/mL) for 30 min at 37 °C. CfDNA was extracted and eluted into 60 μL of elution buffer in three steps (3 × 20 μL) into regular 1.5 mL Eppendorf tubes. In the non-optimized protocol, media w collected and centrifuged at 1000 g and transferred to fresh 1.5 mL Eppendorf DNA LoBind tubes and stored at −20 °C. Before extraction, the medium was thawed at room temperature, and no denaturing agent was added thereafter. CfDNA was then extracted and eluted into 20 μL of elution buffer in one step. Samples were stored in 1.5 mL Eppendorf DNA LoBind tubes. In (**b**) the former experiment was repeated with three new replicates. In (**c**), the average efficacy of the optimized protocols is shown and compared to (**d**), which shows the average efficacy of all variables as determined in the separate experiments. Presumably, the optimized protocol should deliver a value close to this sum total (2.99), but that is not the case. Clearly, some of these changes are working against each other

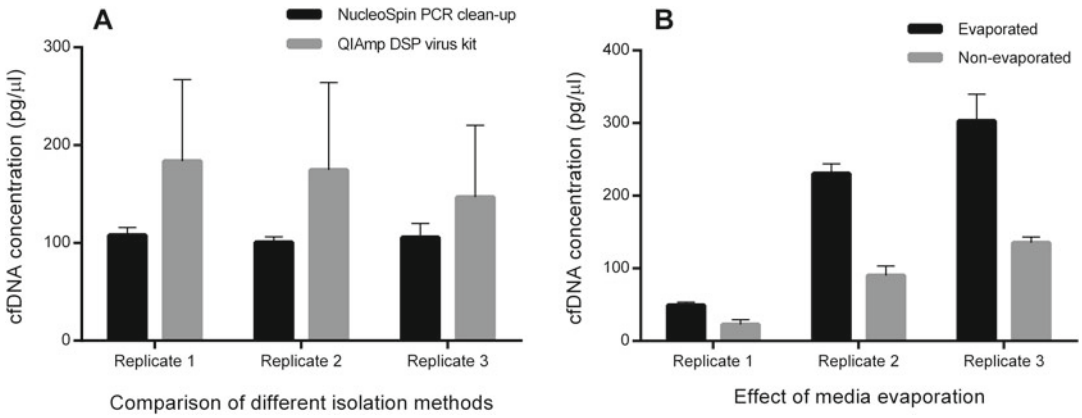

Fig. 29.4 Increasing the yield of cfDNA. (**a**). Comparing the yield of cfDNA delivered by the NucleoSpin PCR clean-up kit and the QIAmp DSP virus kit. The DSP virus kit clearly recovers more cfDNA fragments than the NucleoSpin kit. There was, however, great variation in the yield of cfDNA between the samples in each experiment. This could possibly be due to the non-equal distribution of pressure by the vacuum pump, or some other factor. On the other hand, the NucleoSpin kit was much more consistent. (**b**). The effect of media evaporation. For each replicate, 6 mL of growth medium was aliquoted into 2 mL tubes and evaporated in a SpeedVac to a total volume of 2.5 mL. The cfDNA in these samples were thus expected to be increased 2.4 fold. The average increases of the replicates were then calculated as 2.33 with a standard deviation of 0.2. This is a loss of approximately 3 %, which is rather minor

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Conflict of Interest The authors would like to declare no conflict of interest.

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Novel Technology for Enrichment of Biomolecules from Cell-Free Body Fluids and Subsequent DNA Sizing

30

Vipulkumar Patel, Peter Celec, Magdalena Grunt, Heidi Schwarzenbach, Ingo Jenneckens, and Timo Hillebrand

Abstract

Circulating cell-free DNA (ccfDNA) is a promising diagnostic tool and its size fractionation is of interest. However, kits for isolation of ccfDNA available on the market are designed for small volumes hence processing large sample volumes is laborious. We have tested a new method that enables enrichment of ccfDNA from large volumes of plasma and subsequently allows size-fractionation of isolated ccfDNA into two fractions with individually established cut-off levels of ccfDNA length. This method allows isolation of low-abundant DNA as well as separation of long and short DNA molecules. This procedure may be important e.g., in prenatal diagnostics and cancer research that have been already confirmed by our primary experiments. Here, we report the results of selective separation of 200- and 500-bp long synthetic DNA fragments spiked in plasma samples. Furthermore, we size-fractionated ccfDNA from the plasma of pregnant women and verified the prevalence of fetal ccfDNA in all fractions.

Keywords

Cell-free DNA • DNA size-fractionation • Prenatal diagnostics • Cancer research

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Introduction

The crucial step for an effective genetic analysis of ccfDNA is its isolation in sufficient amounts, even if it is low-abundant and highly fragmented as in plasma (Schwarzenbach et al. 2011). Moreover, for some applications, like prenatal and tumor diagnostics, it may be crucial to separate short ccfDNA fragments from long ones (Chan et al. 2004; Schwarzenbach 2013). Currently, available kits and methods use standard nucleic acid extraction procedures based on sample lysis, binding the nucleic acids on a solid material and washing and elution of nucleic acids. When processing high sample volumes, the procedures are time- and work- consuming, and require large amounts of reagents. Moreover, none of these kits enable to separate ccfDNA in short and long molecules.

We have tested a kit, which differs from other kits by the capturing of biomolecules (DNA, RNA, exosomes, viruses) by a polymer prior to the lysis step and the separation of total ccfDNA in short and long DNA fragments. Large sample volumes can be processed in a fast and simple manner, increasing the probability of capturing low-abundant ccfDNA molecules. The polymer-trapped biomolecules are spun down and can be used either for nucleic acid extraction or can be dissolved in a small volume of a special buffer and used for other downstream applications (e.g. exosome detection by western blot, plaque assay to detect alive viruses). After the nucleic acid isolation, the total ccfDNA (Fraction X) can be selectively size-fractionated in long and short DNA molecules. The size cut-off can be set individually by adding a Binding Conditioner (BC) and should be determined empirically for each application. The amount of BC added to the sample influences the size of long DNA fragments that bind on the spin filter (Fraction Y) and short DNA fragments that pass through the spin filter (Fraction Z). Under special conditions, Fraction Z is subsequently bound to the second filter and both fractions (Y and Z) can be further cleaned up and separately eluted.

This kind of separation allows enrichment of the DNA of interest as well as removal of undesirable DNA, and is qualified for prenatal screening (Ashoor et al. 2013) and cancer research (Schwarzenbach et al. 2012).

Materials and Methods

Extraction and Size Fractionation of Synthetic DNA Spiked in Plasma Samples

Twenty ng of 200- and 500-bp synthetic DNA fragments (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were spiked in 1 ml of plasma samples (Haema, Frankfurt Oder, Germany), extracted and size- fractionated using the PME and subsequent size-fractionized extraction of cell-free DNA kit (Analytik Jena, Jena, Germany). The DNA size cut-off was established by adding 8 µl of BC, resulting in a separation of total DNA (Fraction X) in long DNA fragments (Fraction Y), containing 500-bp DNA, and short DNA fragments (Fraction Z) containing 200-bp DNA. Samples of all three fractions (X, Y and Z) were analyzed on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, California, USA).

Enrichment of Fetal ccfDNA

ccfDNA from 1 ml of plasma from pregnant women bearing a male fetus was isolated and size-fractionized using the PME and subsequent size-fractionized extraction of cell-free DNA kit. The cut-off level was set to 350 bp. Total genome equivalent copies in all three fractions were determined by PCR with primers specific for the ß-globin (autosomal – maternal and fetal) gene. The yield of fetal DNA in all three fractions (X,

Y and Z) was quantified by PCR with primers specific for sex-determining region Y gene.

Results

Extraction and Size Fractionation of Synthetic DNA Spiked in Plasma

The isolated and fractionated synthetic 200- and 500-bp long DNA fragments were analyzed on an Agilent 2100 Bioanalyzer (Fig. 30.1). Total DNA fraction (X) contained equal yields of DNA of both lengths (upper graph). After size fractionation, Fraction Y included almost only 500-bp long DNA (middle graph) and Fraction Z mostly 200-bp long DNA fragments (lower graph). Fractions Y and Z contained negligible traces of 200-bp and 500-bp long DNA, respectively.

Percentage of Fetal ccfDNA in Fractions X, Y and Z

Total genome equivalent copies were calculated in all three fractions of ccfDNA from plasma of pregnant women (Fig. 30.2, red columns). We observed that most ccfDNA was longer than 350 bp. In addition, the yields (green columns) and the prevalence (%, blue columns) of fetal ccfDNA in all three fractions were calculated. The prevalence of fetal ccfDNA was highest in Fraction Z (<350 bp) and lowest in Fraction X (before fractionation). These results indicate the significant predominance of maternal ccfDNA in whole ccfDNA pool.

Discussion

Quantification of low- and high-molecular weight ccfDNA may be of relevance in prenatal screening (Ashoor et al. 2013) and tumor research (Schwarzenbach et al. 2012). Therefore, a fast, standardized and reliable method for size-depending separation of ccfDNA could be used as a diagnostic tool.

Depending on the cancer type, tumor ccfDNA can predominantly be of apoptotic or necrotic origin, and therefore, differently fragmented (Schwarzenbach 2013). Thus, the size-dependent ccfDNA selection could highlight its utility for further genetic and epigenetic analyses.

To date, the clinical relevance and application of ccfDNA have only been found in fetal medicine. Chan et al. (2004) discovered that maternal and fetal ccfDNA differ in their lengths. Low levels of small fragmented fetal ccfDNA are usually detected with a strong background of maternal ccfDNA. It has been reported, that at a 11–13 weeks' gestation, the median prevalence of fetal ccfDNA in total ccfDNA from the mother's plasma is around 10 % and may decrease along with e.g. the mother's origin or body weight (Ashoor et al. 2013). Fetal DNA in maternal plasma has also been shown to be useful for the prenatal diagnosis of certain fetal disorders: fetal chromosomal aneuploidies, sex-linked disorders, cystic fibrosis, Huntington disease and 1-thalassemia (Chiu and Lo 2013). Therefore, the combination of the polymer-based enrichment and subsequent size fractionation of ccfDNA offers a perfect tool for prenatal diagnostics, allowing the enrichment of shorter, fetal ccfDNA by removing longer, maternal ccfDNA from the total ccfDNA fraction.

To our knowledge, this is the first publication showing the separation of ccfDNA in nearly pure short and long ccfDNA fragments. Some faint DNA residues of redundant fractions were observed that should have no significant influence on any downstream applications. However, investigations on large patient cohorts are required to test the clinical relevance of our method.

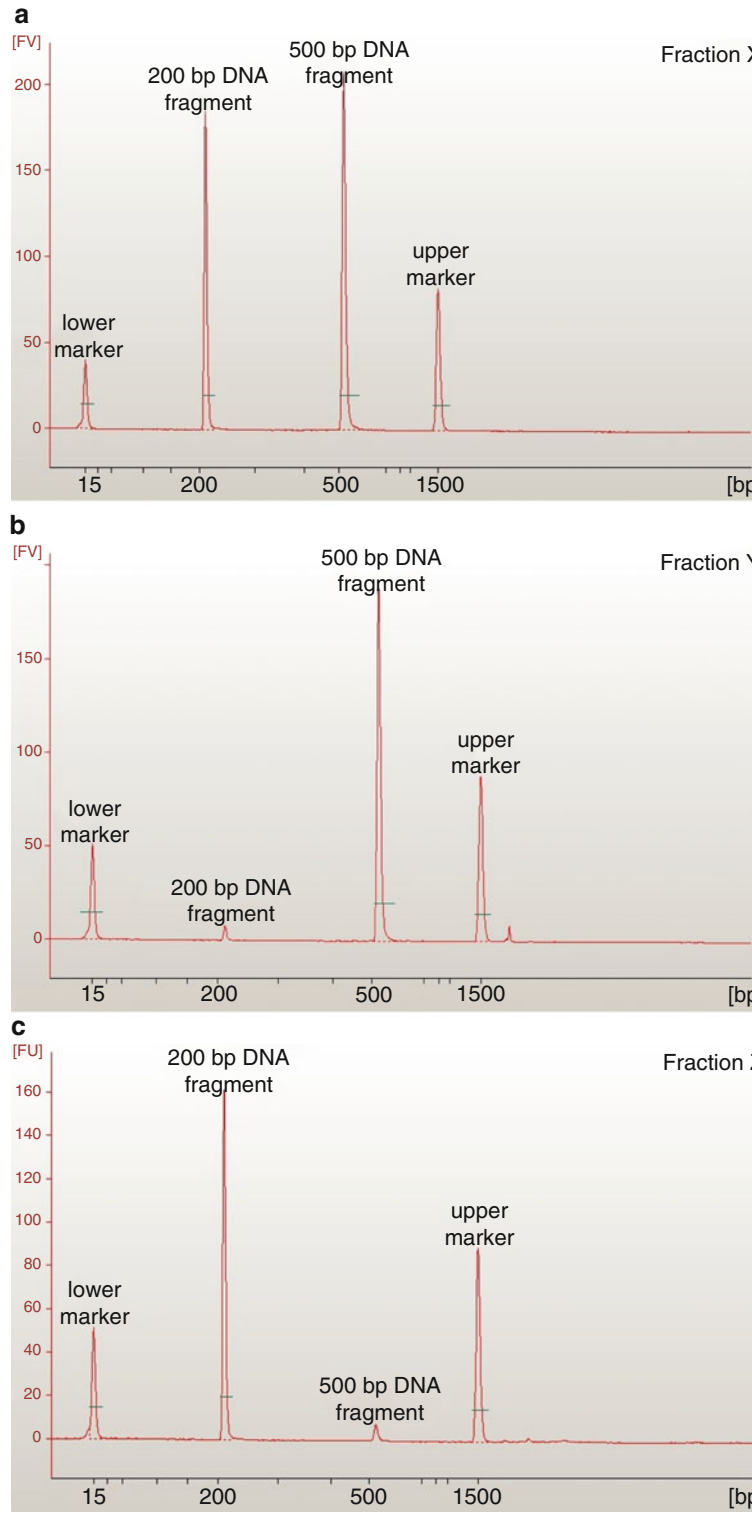

Fig. 30.1 Bioanalyzer examination of isolated and size-fractionated synthetic DNA spiked in a plasma sample; *upper* graph: recovery of DNA of Fraction X; *middle* graph: DNA content of Fraction Y; *lower* graph: DNA content of Fraction Z

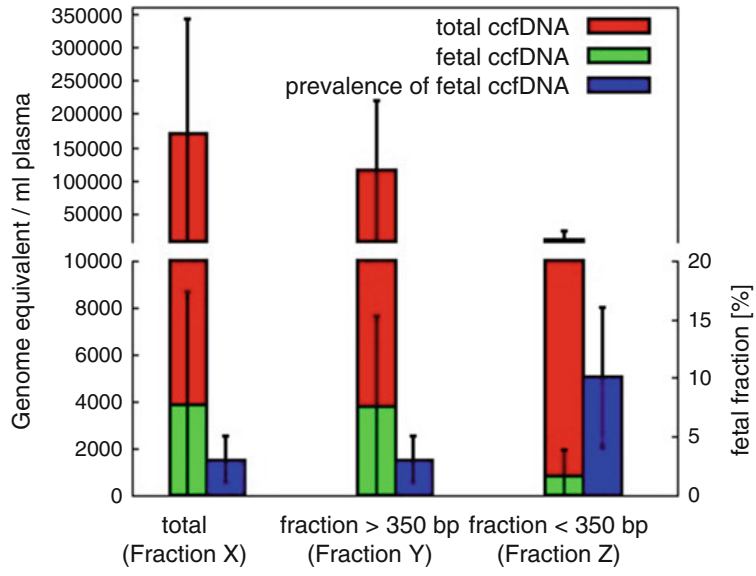

Fig. 30.2 Prevalence of fetal ccfDNA in fractions obtained by size-fractionation; *red*: total genome equivalent determined by PCR targeting the ß-globin gene; *green*: fetal ccfDNA yields determined by PCR targeting sex- determining region Y gene; *blue*: percentage of the fetal ccfDNA in all three fractions

Conflict of Interests No potential conflicts of interest were disclosed.

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A Rapid and Sensitive Method for Detection of the T790M Mutation of EGFR in Plasma DNA

31

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Abstract

Epidermal growth factor receptor (EGFR) T790M mutation is associated with resistance to EGFR tyrosine kinase inhibitors' (EGFR-TKIs) in non-small cell lung cancer (NSCLC). The aims of this study are to develop a blood-based, non-invasive approach to detecting the EGFR T790M mutation in advanced NSCLC patients, using PointMan™ EGFR DNA Enrichment Kit which is a novel method for selective amplification of genotype specific sequences.

Pairs of blood samples and tumor tissues were collected from NSCLC patients with an EGFR activating mutation and who were resistant to EGFR-TKI treatment. EGFR T790M mutation in plasma DNA were detected using the PointMan™ EGFR DNA Enrichment Kit. The concentrations of plasma DNA were determined using quantitative real-time PCR.

Of the 52 patients enrolled in this study, 41 of the patients' plasma samples were collected at post EGFR-TKIs. Nineteen (46.3 %) of the 41 patients had an EGFR T790M mutation in their plasma DNA as detected using the PointMan™ EGFR DNA Enrichment Kit after disease progression to EFGR-TKI. Of 11 cases with a detected T790M mutation from tumor tissues, 10 (90.9 %) also had a detectable T790M mutation in the plasma DNA. There was no difference in the progression-free survival between patients with T790M and those without T790M.

The PointMan™ proved to be a useful method for determining plasma EGFR T790M mutation status

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Keywords
EGFR • EGFRT790M mutation • Non-small cell lung cancer

Introduction

The T790M mutation of *EGFR* occupies about a half of the resistance mechanisms for the first generation EGFR-TKI such as gefitinib and erlotinib in lung adenocarcinoma patients with EGFR activating mutations (Kobayashi et al. 2005; Oxnard et al. 2011; Arcila et al. 2011). Third generation EGFR-TKIs that are expected to have effects for lung carcinoma with a T790M mutation and will be used in the near future (Janne et al. 2015; Sequist et al. 2015). We should examine resistant tumors obtained at re-biopsy to detect if T790M mutation is present. However it is difficult to undergo re-biopsy in some patients because of difficulty in reaching the tumors using bronchoscopy, obtaining the patients' consent and becoming poor performance status. Circulating cell-free DNA (cfDNA) is expected to be an alternative material to detect tumor-derived mutations.

Materials and Methods

Patient Selection and Sample Collection

Fifty-five patients diagnosed as having NSCLC harboring an EGFR mutation were enrolled. The patients were treated with EGFR-TKI therapy and their plasma samples were collected at Kanazawa University Hospital from January 2006 to June 2015. We collected data from their clinical records as follows: age, sex, smoking status, histology, disease stage, EGFR mutation status at initial diagnosis, the agent used as the first EGFR-TKI therapy and the overall response to the first EGFR-TKI therapy.

Plasma Collection

Blood samples were collected from the patients in test tubes containing EDTA before the start of the first round of EGFR-TKI therapy and also at the time of disease progression following EGFR-TKI therapy. DNA solution was extracted from 1 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany).

Detection of EGFR Mutation in Plasma DNA

We used the PointMan™ EGFR DNA Enrichment Kit (provided by EKF Molecular Diagnostics Ltd, Cardiff, U.K.) for the detection of EGFR mutations (Sundaresan et al. 2015). The PointMan™ Assay is a high-sensitivity method for amplifying a gene with specific mutations while inhibiting the wild-type gene. The PCR reaction solutions were visualized using agarose gel electrophoresis to confirm that the target genes had been amplified. After purification of the PCR products, direct sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Survival Analysis

Progression-free survival (PFS) was defined as the interval between the initiation of the first EGFR-TKI therapy and the first manifestation of progressive disease (PD) or death from any cause; patients who were alive and without PD at the time of the analysis were censored.

Statistical Analysis

The $\chi 2$ test was used to analyze the relationships between the T790M mutation status in plasma and several patient characteristics. The age at diagnosis was compared using the Student *t*-test. Kaplan-Meier curves were used to analyze PFS and the log-rank test was used for

Table 31.1 T790M mutation status in the patients

Case #	Tissue post	cfDNA pre	cfDNA post	Case #	Tissue post	cfDNA pre	cfDNA post	Case #	Tissue post	cfDNA pre	cfDNA Post
1	+	+	+	19	n.e.	–	–	37	+	n.e.	+
2	+	–	+	20	n.e.	–	–	38	+	n.e.	+
3	+	–	+	21	n.e.	–	–	39	+	n.e.	+
4	+	–	+	22	n.e.	–	–	40	+	n.e.	+
5	–	+	+	23	n.e.	–	–	41	+	n.e.	+
6	–	–	+	24	–	–	n.e.	42	+	n.e.	+
7	–	–	+	25	n.e.	+	n.e.	43	+	n.e.	–
8	–	+	+	26	n.e.	+	n.e.	44	–	n.e.	–
9	–	+	–	27	n.e.	–	n.e.	45	–	n.e.	–
10	–	–	–	28	n.e.	–	n.e.	46	–	n.e.	–
11	–	–	–	29	n.e.	–	n.e.	47	n.e.	n.e.	+
12	–	–	–	30	n.e.	–	n.e.	48	n.e.	n.e.	+
13	–	–	–	31	n.e.	–	n.e.	49	n.e.	n.e.	+
14	n.e.	+	+	32	n.e.	–	n.e.	50	n.e.	n.e.	–
15	n.e.	–	+	33	n.e.	–	n.e.	51	n.e.	n.e.	–
16	n.e.	–	–	34	n.e.	–	n.e.	52	n.e.	n.e.	–
17	n.e.	–	–	35	n.e.	–	n.e.				
18	n.e.	–	–	36	n.e.	–	n.e.				

Tissue post, re-biopsy tissue sample after progression to EGFR-TKI; *pre* cfDNA before EGFR-TKI, *post* cfDNA after PD to EGFR-TKI, *n.e.* not evaluated

comparisons. All the statistical tests were two-sided, and a P value <0.05 was considered significant.

Results

The T790M status of 52 patients enrolled is shown in Table 31.1. T790M mutations were detected in cfDNA from 19 patients (46.3 %) after progression following the first EGFR-TKI therapy. The incidence of T790M mutation in the cfDNA tended to be higher in men and in patients with an exon 19 deletion. The T790M mutation status at the time of resistance to the first EGFR-TKI therapy was analyzed using tumor DNA derived from the actual tumor tissues in 24 patients (Table 31.2). Twelve (50 %) of the 24 patients had a T790M mutation in the tumor DNA. T790M mutations were detected in cfDNA in 11 (91.7 %) of the patients with the mutation in tumor the DNA and in 3 (25.0 %) of the patients without the mutation. The overall concordance rate was 83.3 % (20/24).

Table 31.2 Relationships of T790M status between re-biopsy tissue sample and cfDNA

		cfDNA		
		Positive	Negative	
Tissue	Positive	11	1	12
	Negative	3	9	12
		14	10	24

In 4 of the 19 patients, T790M mutations were also detected in cfDNA before the EGFR-TKI treatment (Table 31.1). Meanwhile, T790M mutations were only detected before the first EGFR-TKI therapy but not after the detection of resistance, in only one patient.

In the survival analyses, the median PFS of patients with the T790M mutation in their cfDNA was 280 days while that in the patients without T790M was 233 days. No significant difference was observed between the two groups (P=0.60) (Fig. 31.1).

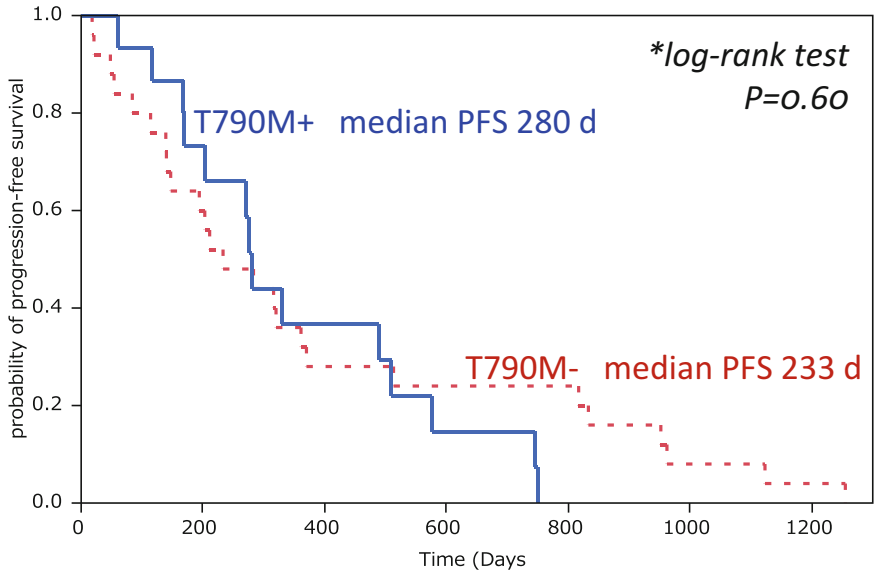

Fig. 31.1 Progression-free survival after first EGFR-TKI therapy in patients with T790M (*solid line*) and those without T790M (*dashed line*) in cfDNA. No statistical difference was observed between the cfDNA T790M mutation status

Conclusion

This assay using the PointMan™ was a useful means of detecting T790M mutations in cfDNA and this study indicates that the mutation statuses of cfDNA and re-biopsy tissue were strongly correlated.

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Evaluation of Different Blood Collection Tubes and Blood Storage Conditions for the Preservation and Stability of Cell-Free Circulating DNA for the Analysis of the Methylated ^{m}SEPT9 Colorectal Cancer Screening Marker

Jurgen Distler, Reimo Tetzner, Gunter Weiss, Thomas König, Anne Schlegel, and Michal Bagrowski

Abstract

For the subsequent analysis of the methylated mSEPT9 colorectal cancer screening marker in plasma, different blood collection tubes and blood storage conditions were investigated. The study demonstrated that methylated Septin 9 (^{m}SEPT9) can be consistently detected in plasma samples derived from whole blood samples collected with S-Monovette® K3E and BD Vacutainer ® K2EDTA tubes stored at 2–8 °C for a maximum of 24 h and for samples collected in S-Monovette CPDA tubes stored at 18–25 °C for up to 48 h.

Keywords

Blood • Stability • Collection tube • Septin 9 • Epigenetic marker • Colorectal cancer

Introduction

The methylated Septin 9 DNA marker is highly correlated with the presence of colorectal cancer (CRC) (deVos et al. 2009). The analysis of ^{m}SEPT9 is performed with free circulating DNA (cfDNA) extracted from human plasma using the Epi proColon® 2.0 CE IVD kit (Epigenomics 2014; Jin et al. 2015). The shipment of blood samples requires the blood to be drawn in

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collection tubes that preserve cfDNA and prevent lysis of blood cells before the plasma can be processed in the laboratory. Ideally, blood collection tubes should allow the shipment of blood at room temperature to avoid complex and costly logistics. The goal of the present studies was to identify a tube enabling the preservation of cfDNA and prevention of lysis of blood cells for at least 72 h at ambient temperature.

Materials and Methods

Blood from male and female healthy donors (age 35–79 years) was collected with different blood collection tubes. To simulate a positive specimen, blood samples were spiked with 15 genome equivalents of HeLa DNA (BioChain Institute, Newark, USA), a genomic human tumor cell line DNA per millilitre blood.

The blood draws with the following collection tubes were performed as recommended by the manufacturers: S-Monovette® K3E (Sarstedt, Nümbrecht, Germany) and S-Monovette® CPDA (Sarstedt, Nümbrecht, Germany), Cell-Free DNA BCT® (Streck, Omaha, USA), PAXgene® Blood DNA Tube (PreAnalytiX GmbH, Hombrechtikon, CH), and BD Vacutainer ® K2EDTA, (Becton Dickinson, Franklin Lakes, USA). Blood collected in BD Vacutainer ® PPT (Becton Dickinson, Franklin Lakes, USA) was not centrifuged directly after blood draw as recommended by manufacturer of the collection tubes, but 24 h later to mimic transport prior to centrifugation of the gel tubes. Subsequently, the centrifuged BD Vacutainer ® PPT tubes were stored.

Blood storage and transport simulation were performed within defined temperature ranges for up to 7 days, the applied conditions are indicated in Table 32.1.

After storage and transport simulation, blood was processed to plasma (Tóth et al. 2014) within 4 h and the targeted ^{m}SEPT9 marker was measured with the Epi proColon® 2.0 CE Assay according to the manufacturers instruction for use (Epigenomics, Berlin, Germany).

The analytical sensitivity (% ^{m}SEPT9 positive samples) and analytical specificity (100 % – % ^{m}SEPT9 positive samples) were determined with plasma of spiked and unspiked blood samples, respectively.

Results

The analytical sensitivity and analytical specificity of the assay using the tested blood collection tubes are summarized in Table 32.1. As reference, plasma of blood collected with S-Monovette® K_3E (Sarstedt) and BD Vacutainer ® K_2EDTA (Becton, Dickinson) tubes without additional storage of blood or after a storage at 2–8 °C for maximum 24 h was analysed. The results derived with the BD Vacutainer® K_2EDTA and S-Monovette® K_3E tubes provided evidence of accurate results with an analytical sensitivity and specificity of 97–100 %.

The alternative blood draw tube S-Monovette® CPDA was successfully tested (positive and negative detection rates of 97 %) for storage time until 48 h at 15–25 °C. With increased storage time for up to 72 h, the analytical specificity was reduced to 89 %.

The blood collected with Cell-Free DNA BCT® tube and the PAXgene® Blood DNA Tube was stored in the tubes for 7 days at 25–30°C prior to plasma preparation. The ^{m}SEPT9 detection rate in spiked plasma obtained from blood collected in Cell-Free DNA BCT® tubes was only 7 %, at an analytical specificity of 93 %. With PAXgene® Blood DNA tubes the positive detection rate for spiked samples was 100 %, with a reduced analytical specificity of 81 %.

In an additional study, a blood collection tube with a gel barrier was tested. The use of BD Vacutainer® PPT tubes resulted in an analytical sensitivity of 83 % and specificity of 89 %, both being reduced in comparison to the reference collection tubes.

Table 32.1 Summary of analysis results of tested blood collection tubes

Blood collection tube[a]	Sensitivity ([m]SEPT9 positive / total samples)	Specificity ([m]SEPT9 positive / total samples)	Blood storage (temperature/time total)
BD Vacutainer® K2EDTA	100 %	100 %	None
(367525)	(36/36)	(0/36)	
BD Vacutainer® K2EDTA	100 %	100	2–8 °C / 24 h
(367525)	(17/17)	(0/17)	
S-Monovette® K3E	97 %	97 %	2–8 °C /24 h
(02.1066.001)	(33/34)	(1/34)	
S-Monovette® CPDA	100 %	100 %	None
(01.1610.001)	(19/19)	(0/18)	
S-Monovette® CPDA	97 %	97 %	15–25°C /48 h
(01.1610.001)	(32/33)	(1/33)	
S-Monovette® CPDA	97 %	89 %	15–25°C/72 h
(01.1610.001)	(37/38)	(4/38)	
Cell-Free DNA BCT®	7 %	93 %	25–30°C/7 days
(218961)	(1/15)	(1/15)	
PAXgene® Blood DNA Tube	100 %	81 %	25–30°C/7 days
(761165)	(17/17)	(3/16)	
BD Vacutainer® PPT	83 %	89 %	25°C/72 h
(362799)	(15/18)	(2/18)	

[a]number indicated: order number of manufacturer

Discussion

The presented data demonstrate that the BD Vacutainer® K_2EDTA, the S-Monovette® K_3E and the S-Monovette® CPDA tubes are suitable to collect blood with the aim of detecting the [m]SEPT9 marker in the plasma. The latter tube allows the storage or shipment of blood at 15–25 °C for up to 48 h.

Blood storage time in S-Monovette® CPDA tubes beyond 48 h at ambient temperature conditions is associated with a reduced specificity of the Epi proColon® 2.0 CE Assay. The Cell-Free DNA BCT® and PAXgene® Blood DNA tubes under the tested conditions are associated with either reduced sensitivity and/or specificity of the Epi proColon® 2.0 CE Assay. The negative impact on the specificity was reproducibly associated with an increased amount of total DNA, possibly derived from the lysis of the peripheral blood lymphocytes (data not shown). The significantly lower sensitivity detected in plasma derived from the spiked blood collected with Cell-Free DNA BCT® tubes is possibly caused by a reduced stability of the cfDNA in this blood samples.

Gel barrier tubes separate blood cells from plasma during centrifugation and it is supposed that a barrier is built to prevent the unintended mixing of cells and plasma during transport at ambient temperature. However, the gel barrier leaks during prolonged storage such that separation of blood cells and plasma was not consistently maintained. Therefore, the use of BD Vacutainer® PPT and S-Monovette® EDTA K2-Gel (initially tested with a very low sample number, data not shown) did not provide the desired results.

Although, the results cannot be applied to other molecular and epigenetic markers to be analyzed in cell-free plasma, the selection of a blood collection tube requires a careful evaluation of the specific parameters for each molecular indication.

Conflict of Interest The authors were employees of Epigenomics AG. Juergen Distler and Thomas Koenig are also share holders of the Epigenomics AG.

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Purification of Circulating Cell-Free DNA from Plasma and Urine Using the Automated Large-Volume Extraction on the QIAsymphony® SP Instrument

Alexander Wolf, Katharina Beller, Sebastian Groemminger, Wera Hofmann, Matthias Sachse, and Jana Fassunke

Abstract

Increasing sample numbers for screening and diagnostics using circulating cell-free DNA (ccfDNA) as analyte demands an automated solution for ccfDNA extraction. The efficiency of a new, automated, large volume ccfDNA extraction method was evaluated against a manual reference method. The new kit for automated ccfDNA extraction on the QIAsymphony showed a comparable yield of total ccfDNA from healthy donors as well as a comparable recovery of circulating cancer and fetal DNA. In conclusion, a new kit for automated ccfDNA extraction was established successfully.

Keywords

ccfDNA • Automation • Fetal • Cancer • QIAsymphony • QIAGEN

Introduction

Circulating cell-free DNA (ccfDNA) is a key analyte for non-invasive prenatal diagnostics and cancer biomarker analysis. Due to extremely low concentrations and a high degree of fragmentation in sample material, the extraction of ccfDNA is technically challenging. Increasing sample numbers requires an automated extraction of ccfDNA. The automated solution must address a high sample input volume to compensate the low ccfDNA concentration while the DNA has to be extracted quantitatively from plasma and eluted

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in a low elution volume to concentrate the DNA. In parallel, instrumentation and chemistry must maintain a high throughput. Here, the efficiency of a new, automated, large volume ccfDNA extraction method was evaluated against a manual reference method.

Materials and Methods

Plasma was obtained by two-step centrifugation from K_2-EDTA tubes and Cell-Free DNA (Streck) tubes. ccfDNA was extracted from either plasma or urine using QIAamp® Circulating NA Kit (QIAGEN, cat. no. 55114) as per the manual reference method and QIAsymphony® Circulating DNA Kit (QIAGEN, cat. no. 1091063) as per the automated extraction method. Urine required an ATL pre-treatment before ccfDNA extraction. ccfDNA yield was determined by the Qubit dsDNA HS Assay Kit (cat. no. Q32851) and real-time PCR (18S, forward primer: 5′-GCCGCTAGAGGTGAAATTCTTG-3′, reverse primer: 5′-CATTCTTGGCAAATGCTTTCG-3′, probe: 5′-ACCGGCGCAAGACGGACCAGA-3′) using the QIAGEN QuantiTect® Multiplex PCR Kit. Size distribution of ccfDNA was determined with the High Sensitivity DNA Kit and DNA 7500 Kit (Agilent). Cancer plasma samples: For the library preparation, an Ion AmpliSeqLibrary Kit 2.0, Thermo Fisher was used. Three nM of each ccfDNA was transferred to a pool that was diluted to 12 pM for targeted NGS. Maternal plasma samples: the fetal fraction was determined using the QuantYfeX® assay and read number in NGS and Z-scores were determined using the PraenaTest® (LifeCodexx AG).

Results

First, size distribution of manually extracted ccfDNA from plasma using the QIAamp Circulating NA Kit was compared to the newly developed automated extraction of ccfDNA using the QIAsymphony Circulating DNA Kit (Fig. 33.1a). Both extraction methods resulted in a comparable size distribution of eluted ccfDNA that showed a curve with a maximum of approximately 165 bp. After pre-amplification, the library preparation ccfDNA showed a more detailed size distribution. The profile revealed, next to the predominant peak, a second and a third peak of fragmented DNA that correlated with a DNA-histone-protein complex and the later distinct peaks match to multi-nucleosomes (Fig. 33.1b). Interestingly, ccfDNA extracted from urine showed a second more fragmented fraction of ccfDNA with a size of approx. 20–100bp (Fig. 33.2a).

Next, total ccfDNA recovery between both the manual and automated extraction method was compared. Yield was calculated by qPCR as target copies per milliliter sample. Both methods clearly showed strong individual variations in ccfDNA concentration in plasma and urine from healthy donors typically observed within a population (Figs. 33.2b and 33.3a). Overall the QIAsymphony Circulating DNA Kit showed a comparable extraction efficiency to the QIAamp Circulating NA Kit.

To show linearity of ccfDNA extraction about a broad range of plasma input, we compared the 2 ml and 4 ml protocol with a customized 6 ml protocol. DNA yield was determined by real-time PCR using three different input volumes 2, 4 and 8 µl (Fig. 33.3b). A linear increase of ccfDNA yield was observed for 2, 4 and 6 ml plasma input. Moreover, the detected copy numbers of ccfDNA slightly increase if the PCR input volume increases from 2 to 8 µl indicating that due to the very low DNA concentration in the eluate, PCR efficiency increases if more template is offered to the polymerase.

Finally, we confirmed that the generic sample extraction on the QIAsymphony shows compatibility to downstream assays customers use. Compatibility to clinical cancer samples was shown with regard to total ccfDNA yield determined by Qubit dsDNA HS Assay Kit (Fig. 33.4a). A comparable mutation frequency for the QIAamp Circulating NA Kit and

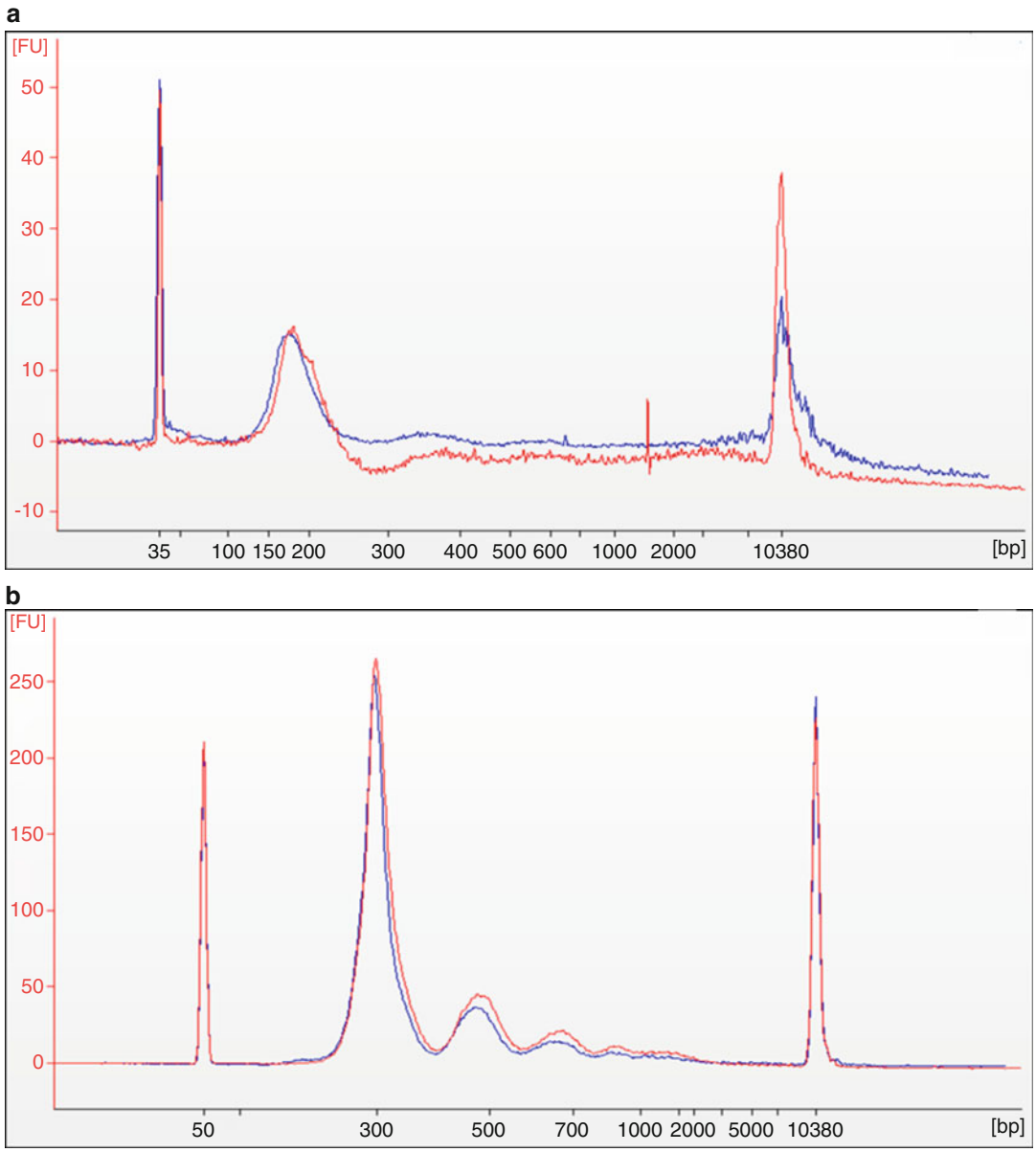

Fig. 33.1 Size distribution of extracted ccfDNA from plasma. ccfDNA extracted from 4 ml maternal plasma using QIAamp Circulating NA Kit (dark grey) and QIAsymphony Circulating DNA Chemistry (light grey). One µl eluate subjected to Agilent High Sensitivity DNA Kit (5–500 pg/µl) (**a**). Five ng eluate subjected to library prep. One µl purified eluate from library prep subjected to Agilent DNA 7500 Kit (**b**)

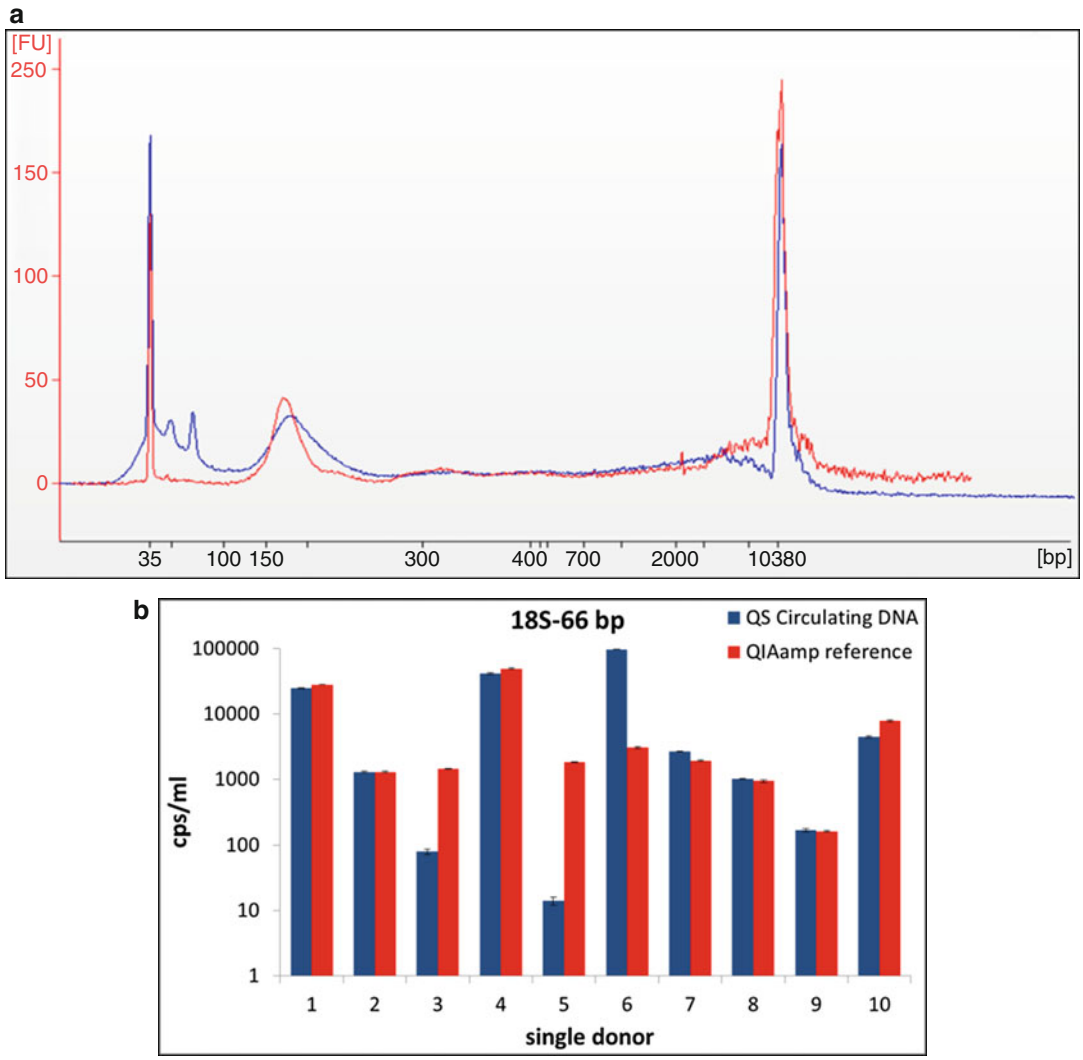

Fig. 33.2 Extraction of ccfDNA from 4 ml urine. (**a**) ccfDNA extracted from 4 ml urine (dark grey) and plasma (light grey) using QIAsymphony Circulating DNA Chemistry. One µl eluate subjected to Agilent High Sensitivity DNA Kit (5–500 pg/µl). (**b**) Urine samples from ten healthy donors; 3–4 ml urine as sample input. Circulating DNA yield determined by real-time PCR (18S). Results were calculated as target copies per ml plasma

QIAsymphony Circulating DNA Kit was calculated from individual donors using targeted NGS (Fig. 33.4b). Next, compatibility to maternal plasma samples was evaluated for Non Invasive Prenatal Diagnostic. Eluates containing extracted ccfDNA were subjected to the QuantYfeX® assay to determine the fetal fraction and to NGS. The results clearly show that the fetal fraction is increased for the new ccfDNA extraction on the QIAsymphony (Fig. 33.5). The percentage of unique and mapped reads is comparable between both methods. Z-Scores for chromosome 13, 18 and 21 are comparable around zero as expected for healthy euploid sample material. A z-score of three is the cut-off, which would indicate an aneuploidy or trisomy. In conclusion, the NGS data conform to the PraenaTest® quality standards for both the QIAsymphony and QIAamp ccfDNA extraction methods (Fig. 33.5).

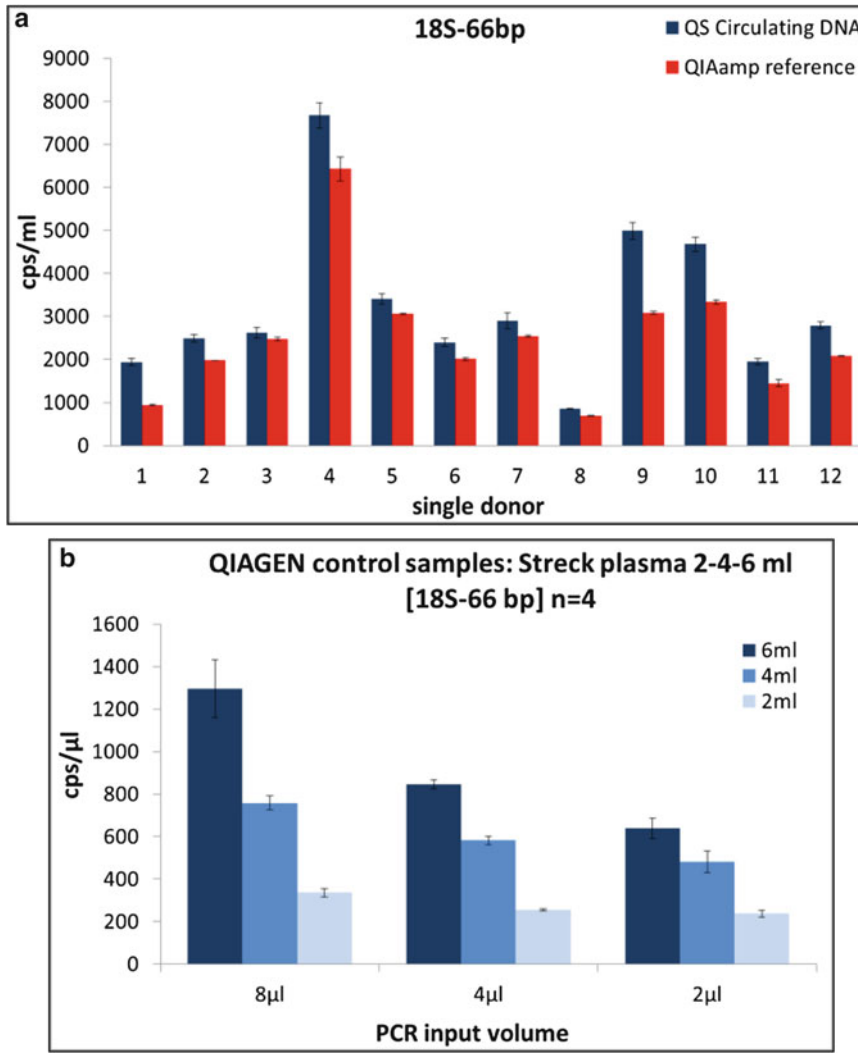

Fig. 33.3 Extraction of ccfDNA from 2–6 ml plasma. Plasma obtained from 12 healthy donors in Cell-Free DNA tubes (Streck). (**a**) ccfDNA extraction from 4 ml plasma using QIAamp Circulating NA Kit and QIAsymphony Circulating DNA Chemistry. Circulating DNA yield determined by real-time PCR (18S). (**b**) ccfDNA extraction from 2 ml, 4 ml and 6 ml plasma using QIAsymphony Circulating DNA Kit. Circulating DNA yield determined by real-time PCR (18S) using three different PCR input volumes (2-4-8 µl). Results were calculated as target copies per µl eluate

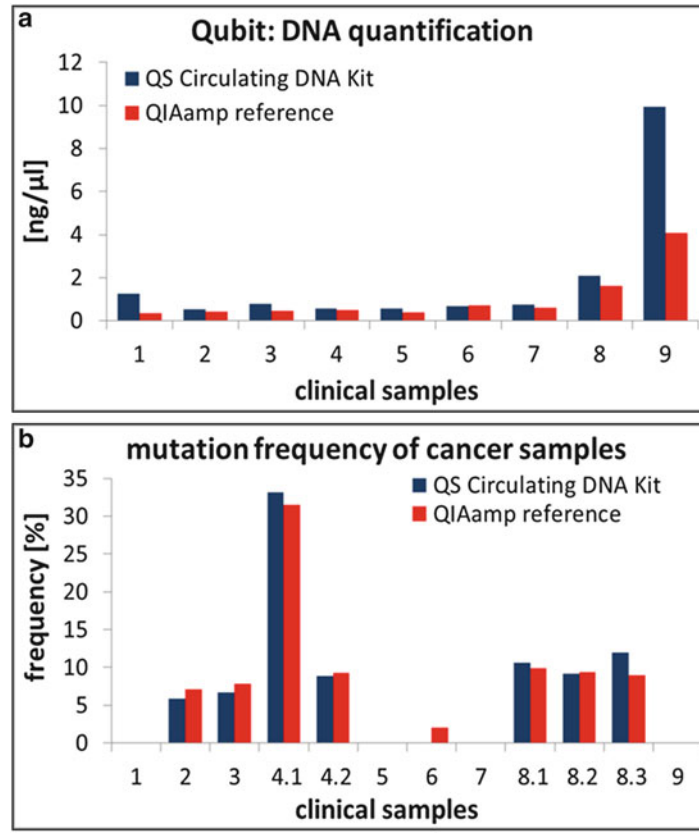

Fig. 33.4 Detection of cancer ccfDNA from clinical samples. Plasma samples from nine clinical donors. ccfDNA extraction from 3.0 to 3.5 ml plasma using QIAamp Circulating NA Kit and QIAsymphony Circulating DNA Kit. (**a**) Circulating DNA yield determined by Qubit dsDNA HS Assay Kit. Results were calculated as ng DNA per µl eluate. (**b**) 12 µl eluate was subjected to library prep and subsequent targeted NGS. Results were calculated as mutation frequency (1:w/o, 2:TP53 exon5, 3:KRAS exon2, 4.1:EGFR exon19, 4.2:EGFR exon20, 5:w/o, 6:EGFR exon19, 7:w/o, 8.1:EGFR exon21, 8.2:EGFR exon20, 8.3:TP53 exon3, 9: w/o)

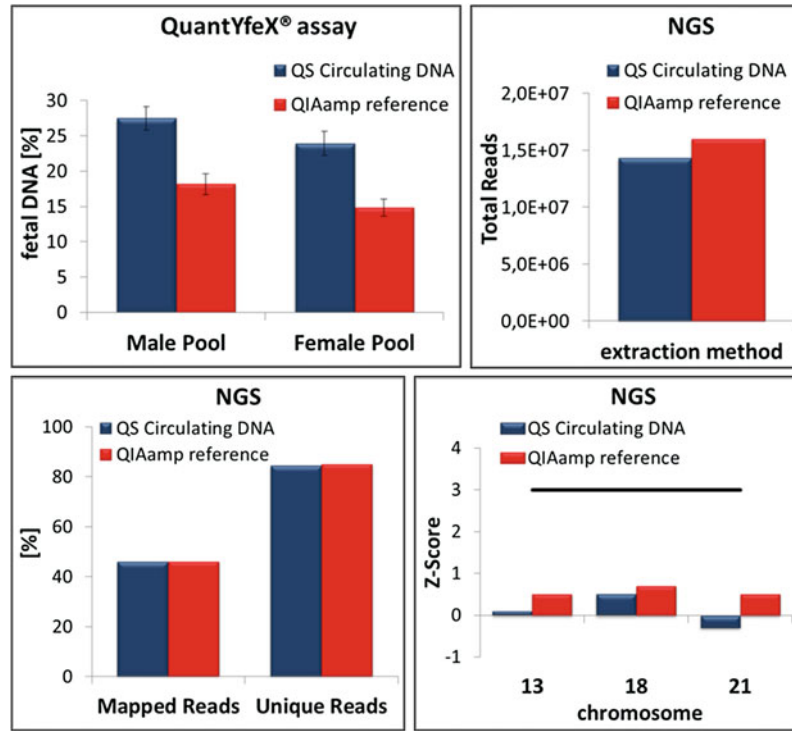

Fig. 33.5 Detection of fetal ccfDNA fraction in maternal plasma. ccfDNA extracted from 4 ml maternal plasma (female/male fetal ccfDNA) using QIAamp Circulating NA Kit and QIAsymphony Circulating DNA Kit. Eluates subjected to QuantYfeX® assay to determine the fetal fraction and to NGS to determine Z-score (PraenaTest®)

Discussion

The presented results show that the QIAsymphony Circulating DNA Kit in combination with QIAsymphony SP instrument is a suitable solution for automated ccfDNA extraction. The novel protocol enables automated ccfDNA recovery from up to 4 ml plasma and up to 96 samples per run in 6 h combined with high recovery efficiency of total ccfDNA as well as cancer and fetal ccfDNA.

The applications presented here are for research use only. Not for use in diagnostic procedures.

Conflict of Interest Work presented here was funded by QIAGEN's R&D Department. A. Wolf and K. Beller are employees of QIAGEN GmbH. S. Groemminger, W. Hofmann and M. Sachse are employees of LifeCodexx AG. J. Fassunke is an employee of Universitätsklinikum Köln.

Detection and Quantification of KIT Mutations in ctDNA by Plasma Safe-SeqS

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Johannes Fredebohm, Daniel H. Mehnert, Ann-Kathrin Löber, Frank Holtrup, Vanessa van Rahden, Philipp Angenendt, and Frank Diehl

Abstract

We have designed a highly sensitive assay based on the Safe-SeqS technology to detect *de novo* mutations in the KIT gene and tested its performance. This assay was applied to plasma samples of GIST patients before and after treatment with a multikinase inhibitor and mutations at known and novel sites of potential secondary resistance were identified.

Keywords

ctDNA • KIT • Safe-SeqS • Secondary resistance • GIST • NGS

Introduction

Gain-of-functions mutations in the KIT gene are frequently detected in gastrointestinal stromal tumors (GIST). These primary mutations cluster in specific domains and play an important role in tumor development. Furthermore, secondary mutations have been identified that only occur during treatment with targeted therapies and vary between patient and metastatic site. Determining these secondary resistance mutations is limited by the need to have serial tissue samples available from representative metastases, since the genomic heterogeneity of secondary mutations is poorly represented in single tissue specimens. For this reason, we sought to develop a ctDNA based sequencing assay that covers the most relevant regions of the KIT gene.

Methods

We have developed a KIT sequencing assay based on the **Safe-Seq**uencing **S**ystem (Safe-SeqS) (Kinde et al. 2011) (Fig. 34.1). Specifically, two multiplex primer panels were established to cover all hotspot mutations (COSMIC, Forbes et al. 2015) and all known secondary resistance mutations

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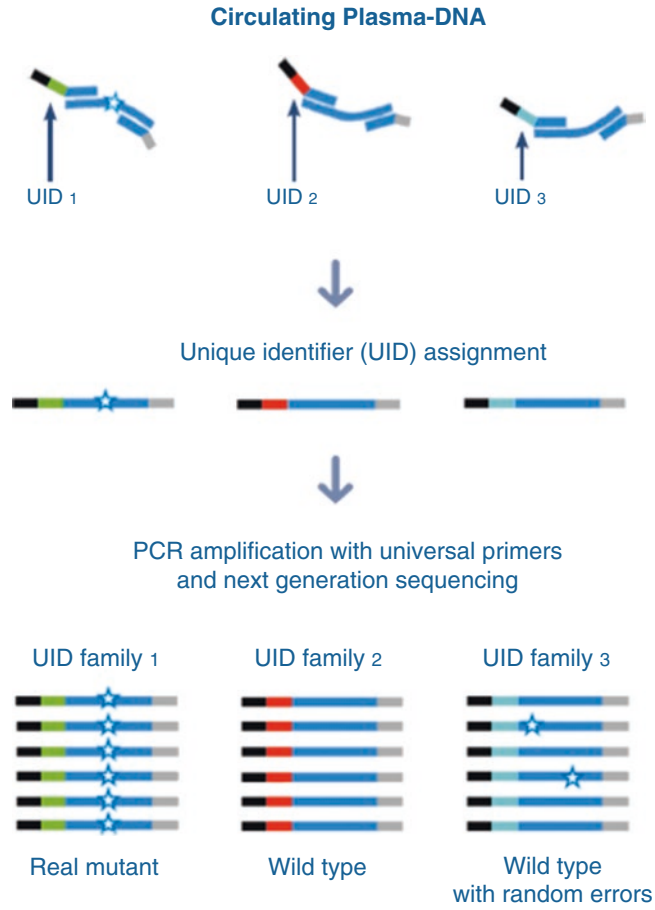

Fig. 34.1 Plasma Safe-SeqS assay principle. Generation of unique identifier (UID) families followed by standard NGS allows discrimination between real mutants and random errors introduced during the library preparation and sequencing. UID-barcodes are added during a first PCR amplification. UID families are generated in a second PCR

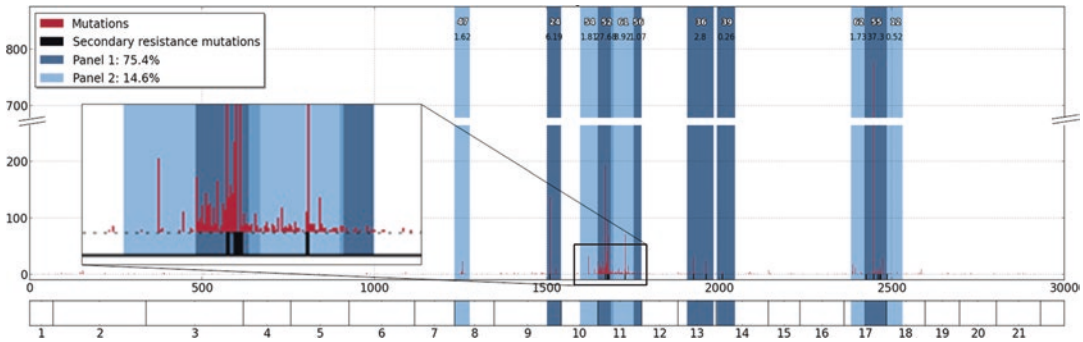

Fig. 34.2 Mutation coverage of the KIT by the Plasma Safe-SeqS assay. Two panels have been developed that cover all hotspot mutations (COSMIC, Forbes et al. 2015) and all known secondary resistance mutations

(Fig. 34.2). Plasma from healthy donors as well as lymphocyte DNA spiked with synthetic DNA was utilized to establish analytical performance. Furthermore, plasma from advanced GIST patients enrolled in a clinical trial was tested with the Plasma Safe-SeqS technology.

Results

The performance of a KIT ctDNA sequencing assay using the Safe-SeqS technology was assessed. Spike-in controls were used to demon-

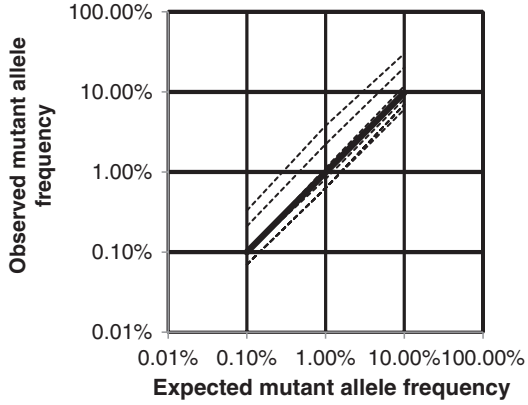

Fig. 34.3 Genomic DNA samples from lymphocytes spiked with synthetic DNA carrying specific mutations were quantified in triplicate by a PicoGreen® assay and used to demonstrate accuracy of Plasma Safe-SeqS assay in an allele frequency range from 0.1 to 10 %

strate the quantitative nature of the KIT PSS assay (Fig. 34.3). While accuracy was high with an average on-target rate of 98.9 %, precision was low due to variation in quantification of artificial DNA by the PicoGreen® assay (CV = 33 %).

The PSS KIT assay was able to identify novel secondary resistance mutations. Ninety six GIST patients previously treated with imatinib and sunitinib were sequenced at enrollment. At enrollment, a total of 120 already known single base variations were observed in addition to 54 previously unknown variations. Thirty three of these patients were also sequenced at end of treatment. Between these two time points a decrease of secondary resistance mutations was observed for 22 known and three novel ones. In other patients, mutant fractions increased during treatment. Among these were 14 known and four novel mutations.

Discussion

We have developed a highly sensitive Plasma Safe-SeqS assay to identify KIT mutations in ctDNA. Our data suggests that this sequencing assay enables reliable *de novo* detection of sequence variations in ctDNA isolated from plasma. It may be applied to the genotyping of advanced GIST tumors prior to treatment as well as subsequent monitoring of resistance mutations in patients receiving targeted therapy. We demonstrate here the detection of novel putative secondary resistance mutations that make up about one quarter of all mutations detected in this study.

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Part V

Ethics

Lost in Translation? Ethical Challenges of Implementing a New Diagnostic Procedure

35

Dagmar Schmitz

Abstract

Since cell-free DNA (cfDNA) fragments of placental origin can be isolated and analyzed from the blood of pregnant women. Applications of this finding have been developed and implemented in clinical care pathways worldwide at an unprecedented pace and manner. Implementation patterns, however, exhibit considerable insufficiencies. Different "motors" of implementation processes, like the market or various regulatory institutions, can be identified at a national level. Each "motor" entails characteristic ethical challenges which are exemplified impressively by a rising number of case reports.

Empirical data demonstrate that there are significant "losses" in the respective translational processes, especially when the results from clinical research are to be translated into the clinical reality of NIPT (the so called "second roadblock" (T2)). These "losses" are perceived in the fields of knowledge transfer, professional standardization and ethical debate. Recommendations of professional organizations often fail to reach general practitioners. Blindsided by the new diagnostic procedure in their clinical practice, professionals in prenatal care express their insecurities with regard to its handling. Ethical debate appears to adhere to pre-existing (and partly already proven to be insufficient) normative frameworks for prenatal testing. While all of these deficits are typical for the implementation processes of many new molecular diagnostic procedures, especially in NIPT, they show a high variability between different nations.

A critical assessment of the preferred strategy of implementation against the background of already existing national ethical frameworks is indispensable, if potential adverse effects are to be diminished. The

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described translational losses seem to be significantly reducible by granting the translational process in roadblock T2 more time.

Keywords
Prenatal diagnosis • Non-invasive prenatal testing • Translational research • Professional ethics

Introduction

At a first glance, the implementation of non-invasive prenatal testing (NIPT) procedures on the basis of cfDNA impresses as a success story. It took only 10 years from the description of cfDNA of placental origin in the maternal blood (Lo et al. (1997)) to the proof-of-principle for the use of next generation sequencing for the detection of fetal aneuploidies in NIPT (Lo and Chiu 2008; Fan et al. 2008). In translational research, which is directed towards improving "bench-to-bedside" processes in medicine, this step is also referred to as the first translational roadblock (T1) (Woolf 2008; Sung et al. 2000). More recent definitions differentiate even more and for example distinguish between the stages of basic, preclinical and clinical research, clinical implementation and public health (https://ncats.nih.gov/translation/spectrum; Schwartz and Vilquin 2003). The second roadblock, in contrast, comprises efforts to translate "the results from clinical studies into everyday clinical practice and health decision making" (Sung et al. 2000). Here, the example of NIPT stands for an even faster development. In 2011, the first providers offered NIPT in China and the US. Four years later, NIPT is available in more than 60 countries worldwide (Allyse et al. 2015) with considerably more than 500,000 tests having been sold (Chandrasekharan et al. 2014). NIPT is described as a "high-performance race car" (Bianchi and Wilkins-Haug 2014) with the pace of advancement in this field being "extremely rapid" (Everett and Chitty 2015). At the same time, a growing awareness with regard to the side effects of such a pace of implementation is perceptible. The need to "urgently address how we structure our services so that we can provide safe services" (Everett and Chitty 2015) is expressed. Increasingly, case reports are published which illustrate significant shortcomings of the implementation process (Verweij et al. 2014). After a few words about translational processes and its motors, the main areas of translational losses for NIPT procedures will be analyzed and illustrated exemplarily.

Translational Highways

Translational research seems to be a field of all-embracing importance in medicine. Countless initiatives are providing support, funding and awards in this research area. The National Institutes of Health (NIH) for example has established the National Center for Advancing Translational Sciences (NCATS) in 2012 with the intent "to reduce, remove or bypass costly and time-consuming bottlenecks in the translational research pipeline in an effort to speed the delivery of new drugs, diagnostics and medical devices to patients" (https://ncats.nih.gov/about/center). Translational research programs are supported by patient representative organizations as well as by the industry. The Seventh Framework Programme (FP7; 2007–2013) of the European Union had translational research as one of its main objectives. The European Advanced Translational Research Infrastructure in Medicine (EATRIS), which comprises more than 70 academic institutions across Europe, has been launched in 2013 in order to bring "drugs, vaccines and diagnostics into common patient usage" (http://horizon-2020projects.com/excellent-science/eartis-will-bridge-the-valley-of-death/). In spite of all these initiatives, the experienced gap between basic or clinical research on the one hand and health gains

for the public on the other hand is still a major one, the "valley of death" (Butler 2008) still not bridged. One suspected cause is, until now, an insufficient recognition of the distinctive demands and problems in the translational roadblock T2 which also is displayed by less funding than for T1 (Woolf 2008). The disciplines involved in T2 are different (clinical epidemiology, communication theory, public policy and the like) and so are the supportive needs.

That these are widely not met so far is especially evident in NIPT. An analysis of translational processes in various countries regarding the implementation of NIPT in the overwhelming majority reveals very few similarities to the often-cited ideal of a "translational highway". A well-planned and founded, solidly built structure to bridge the theory-practice gap and allow fast and safe translation of information is missing. Depending on the respective national motor of implementation, the resulting processes recall either a fixed-rope route (mainly market-driven) or a hastily built suspension bridge (driven by various internal/professional regulatory institutions). Instructive exceptions to this rule can be examined for example in The United Kingdom and The Netherlands, where both have set up elaborate research programs and regulations in order to accompany and structure implementation processes and maximize health gains for their societies (van Schendel et al. 2015; Hill et al. 2014).

Translational Losses: A Case Report

In Germany, as well as in many other countries (among them the USA), the process of implementation has been mainly market-driven in the beginning. It is the major advantage of this way of implementation that it is quickly set up, the roadblock T2 in this case short-term available, which means that very early after the first clinical studies pregnant women had the possibility to choose NIPT in prenatal testing. Like a fixed-rope route, however, it has certain disadvantages. In particular, it is limited in its accessibility and safety. Important information from both sides might get lost in the process or even excluded from translation in advance. A short case example from 2014 (when NIPT has been available in Germany for more than 1 year) might illustrate these limitations (Reported in GebFra – currently under review).

A 36-year-old woman in her second pregnancy repeatedly asked for prenatal testing, especially for NIPT, but was always told that she is still young and so does not need to worry. As she insisted, however, her gynecologist agreed to take a blood sample in the 12th week and to send it to a US provider for NIPT. She received no counseling on the possibilities and limitations of NIPT or other procedures of prenatal testing at all. In the following weeks, she several times called her gynecologist, but was always told, that the results are still pending. In her 18th week, she was finally told that the results were lost, but her gynecologist did not offer her any alternative possibilities in prenatal testing. By chance, 2 weeks later he saw a heart malformation with ultrasound. She then went to an MFM specialist and the following tests revealed a trisomy 13 in the fetus.

Three Areas of Translational Losses

The case example highlights losses of the translational process in three different areas – knowledge, professional standards and ethical debate. Firstly, the gynecologist obviously lacked the relevant *knowledge* in order to manage the testing and provide adequate genetic counseling. He neither informed her about the necessity to verify pathological findings in NIPT via invasive testing nor about the limitations of NIPT. First empirical studies now show that this is a common problem in the clinical handling of NIPT. Obviously, a substantial part of clinicians and even many MFM specialists (13 % in one study (Haymon et al. 2014)) offer NIPT falsely for diagnostic testing. In one study, 6 % of the women with pathological findings in NIPT terminated the pregnancy without karyotype confirmation (Dar et al. 2014) that might be one possible consequence of inadequate counseling.

Professional *standards* for diagnostic or therapeutic procedures are developed in order to safeguard their clinical use, to inform physicians and protect patients. In Germany, and many other countries, there are no standards for the clinical use of NIPT so far. Otherwise, in all likelihood the presented case would have taken another course. Several aspects do impede professional standardization. The necessary medical and structural knowledge is still incomplete. We only now are learning more about incidental findings in NIPT (Bianchi et al. 2015) and structural consequences for the clinical care pathways in prenatal testing (Warsof et al. 2015). There is, however, also a long-known deficit in cooperation between the involved professional organizations in prenatal care, which is especially visible when new diagnostic procedures like NIPT demand a rapid response.

Thirdly, and maybe most importantly, the presented case exemplifies an *insufficient debate of the professional ethics* in relation to prenatal testing and NIPT. The professional actions of the gynecologist exhibit a personal moral uneasiness with prenatal testing, which at least partly reflects unsolved problems of professional ethics in prenatal testing. NIPT is not posing them with a new quality, but rather with a heightened impact. The established normative framework of reproductive autonomy and choice has been proven insufficient already and is now leading pregnant women in an "information overload" (de Jong et al. 2010). A new potential framework, however, needs time in order to evolve. Furthermore, the debate is characterized by many blind spots, like the issue of the fetus as a second patient in prenatal care. A substantial and multidisciplinary effort is needed if gynecologists as well as pregnant women shall no longer be left alone with the intrinsic ethical difficulties of prenatal testing.

Summary

It takes time to build the often called for translational highways for bridging the theory-practice gap and bringing new technological advances to the patient. Fast-track translational solutions might seem to be an appealing alternative, especially for the second translational roadblock. They, however, come along with significant losses in the areas of knowledge, standardization and ethical debate. The example of NIPT teaches us that the prize, which pregnant women have to pay for these time-saving translational solutions, is too high.

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Part VI

Round-Table

Academia Meets Industry

36

Christian Schäfer, Tobias Paprotka, Ellen Heitzer, Mark Eccleston, Johannes Noe, Stefan Holdenrieder, Frank Diehl, and Alain Thierry

Abstract

Researchers working in industrial laboratories as well as in academic laboratories discussed topics related to the use of extracellular nucleic acids in different fields. These included areas like non-invasive prenatal diagnosis, the application of different methods for the analysis and characterization of patients with benign and malignant diseases and technical aspects associated with extracellular nucleic acids. In addition, the possibilities and chances for a cooperation of researchers working in different worlds, i.e. academia and industry, were discussed.

Introduction: Welcome to Our Today's Panel Discussion on "Academia Meets Industry"

And these are our guests today:

Tobias Paprotka Tobias earned the Salzman Memorial Award for his research at the HIV Drug Resistance Program of the National Cancer Institute-Frederick. He is currently working for GATC Biotech, a leading service provider on DNA and RNA sequencing made in Germany. He is responsible for RND, currently focusing on circulating DNA methods.

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Ellen Heitzer Ellen is Assistant-Professor at the University of Graz. After a research visit to the Welcome Trust Centre of Human Genetics at Oxford University, she was responsible for two research projects on therapy monitoring of colorectal and prostate cancer.

Mark Eccleston Mark is an entrepreneur with extensive experience in the biotechnology sector, both in academia and in industry. He holds management roles at Volition in Belgium and Singapore and at OncoLytika in the UK. He sees his main focus in product innovations in the field of diagnostic and therapeutic applications.

Johannes Noe Johannes is a biologist by training and spent his post doc in Zurich doing research on drug transporters. He is now working for Roche Genentech in Basel as leader of companion diagnostics with a focus on phase 3 studies in oncology and non-oncology.

Stefan Holdenrieder Stefan Holdenrieder started his clinical career at the University Hospital in Munich and is now working as a senior physician at the Institute of Clinical Chemistry and Clinical Pharmacology at the University Hospital in Bonn. His main research focus is the development and evaluation of new biomarkers for the early diagnosis, therapy stratification and monitoring of different types of cancers.

Frank Diehl Frank started his academic career at the German Cancer Research Center (DKFZ) in Heidelberg and at the John Hopkins University in Baltimore. He co-founded the company Inostics. He now works as the Chief Scientific Officer at SYSMEX Inostics.

Alain Thierry Alain is the Research Director at the National Institute for Health and Medical Research in Montpellier. He has extensive experience in cancer therapy and drug delivery, was founder of the biotech company MedInCell and was Associate Professor at the University of Montpellier. He has just founded the company DiaDX.

Christian Schäfer So, Welcome from my side to tonight's panel discussion and my first question is to the audience: So, who is from Academia and who is from Industry? Please raise your hands: Academia? Industry? Wow, OK, it was almost, from our side, 50:50.

Christian Schäfer Stefan. Have you ever thought of switching positions to the Industry side?

Stefan Holdenrieder Yes, frequently. [Laughing] No, I think there are very good opportunities in Industry to develop a product from the beginning to the end and bringing it to a quality where it can be used for patient care. I think that this is a very nice field in which to work and to bring a medical input and needs; this would be quite challenging. But to be honest, at the moment, the academic freedom is more important for me so that I can do what I want – and so this is my choice.

Christian Schäfer Could you define academic freedom for us?

Stefan Holdenrieder Yes, I have seen sometimes, that even if there was a product which was relevant and useful for patients, this was not developed any further because of political reasons. So I think that if I see a need, a medical need, then I really would like to try something that I think could help to meet this need.

Christian Schäfer Frank, what brought you from the academic side to being an entrepreneur?

Frank Diehl To the dark side… [laughing] So, initially when we did this research at Hopkins, I approached companies at the time – diagnostic companies, and I heard this also on a talk earlier, people looked at me and said: "You're crazy. There is no DNA. What is this?" You know, people knew about CTCs, but circulating DNA was certainly not so prominent in large diagnostic companies and really what we thought was to take this academic research to clinical application; and the only way to do it is to commercialize and to professionalize it. And that's why then we decided to start a company ourselves.

Christian Schäfer And did you bring the academic freedom into the company or do you still have this academic freedom?

Frank Diehl That's also a good point. Initially, certainly we had some freedom, but quickly we also had to also focus to keep a revenue stream, to stay alive as a company. As I mentioned, investors at the time didn't realize what this is all about, it was more like a crazy idea. So now you talk to investors and they know about it, so, you quickly have to learn to be efficient.

Christian Schäfer Alain, another true entrepreneur as I learned yesterday in our initial talk. Can you tell us a little more about your newest company? Is this a spin-off of your institute?

Alain Thierry No. I'd first like to indicate that I have a first life in science because I first worked on gene delivery transfer and this field went to circulating DNA, when I worked at the Institute of Diagnostics. So, I was a co-founder of a company on drug delivery before, on liposome delivery of genes and oliogonucleotides. I quit this company and I became associate professor before entering this institute of diagnostics and I followed the work of Frank and I thought this is a crazy idea that I'd like to work on. I think it is a very exciting field and I worked first on the fundamental area of the structural origin of circulating DNA and then I always applied studies and fortunately we wrote a patent and from the patent it's also logical to link the patent to a company. And this company DiaDX is a company devoted to circulating DNA and cancer.

Christian Schäfer Mark, I was amazed about the marketing presentation today from a science guy. So how much science guy are you still and how much of a marketing and business guy are you?

Mark Eccleston The advantage of small companies is that everybody does a lot of different things, but I think at heart I'm still a scientist and that helps on the commercial side because you have a better understanding of what you're talking about. I've been in Academia for 10 years, following my PhD, and I got the opportunity to do an MBA while I was doing that through an enterprise fellowship scheme which was designed to turn a scientist into, or at least to equip them to deal in, the commercial world. So that got me very interested in that side of things. So, that essentially led me to the dark side I guess, but also I was – as Stefan was saying – you know the freedom to do what you want – I was looking for financial freedom within research essentially and I became frustrated with the grants schemes that we are looking at through the academic side. And, I got frustrated looking at the venture capital side of things because, you know, constantly trying to raise money and get it, and you know it was just never there, so, actually working with a company that manages to raise the money that we required to go forward through the stock market opened my eyes to the possibilities basically as a very interesting process. There is a focus required, as you said, on the commercial side, but actually always looking slightly forward so I have all the people in the company to kind of ring me back in and say "OK this is what we're doing at the moment", so this is what you can do to help them to promote and to sell that. But, also, in terms of looking forward into the future I guess I'm always thinking 2 or 3 years ahead of what's gonna happen next in terms of the science, but also in terms of the products we can roll-out.

Christian Schäfer Ellen would the Industry be an option at all for you?

Ellen Heitzer To be honest: No. I'm a hundred percent science, academia guy. Well, its actually not totally true because I work at the Institute of Human Genetics and there I'm involved in the routine diagnosis of hereditary diseases, so I'm in charge of the diagnosis of hereditary tumor syndromes, and although we do this from the academia point of view, so we are not a company, it's still done by the University,. We earn money with this diagnosis, but as for the liquid biopsy, I like to be independent and try new things whenever I want. But, I agree with what Frank said before, if you really want to put the liquid biopsy forward into the clinical routine, then you can't just stay at this exploratory level, so, for me, I'm happy with Academia.

Christian Schäfer Tobias, again a very marketing-oriented presentation I saw earlier. How close is your CFO controlling you on research topics? How free are you?

Tobias Paprotka It's actually, we of course work very closely together, but I would say we develop the ideas together and especially for the liquid biopsy field. As we have seen today, there are so many possibilities so sometimes it is very hard to focus; so I would say we have extensive discussions and in the end we always agree.

Christian Schäfer Johannes, the Genentech guy, the Roche guy and we had a brief talk before this round. How is life at Genentech compared to Roche?

Johannes Noe So, there is not much difference, I must say, so, in principle, let me phrase it this way. I'm located in Basel, and I belong to a group in Genentech where all my colleagues are sitting in Genentech. I have a lot of interaction with them and you can imagine that a 9-h time difference is not always easy. But, there is not so much difference between the two companies as such, right, so we are all striving for developing drugs and we are developing, in my case, companion diagnostics with the drugs. That's something which is really rewarding, I think, because I went through this company in various positions, and I started from the University Hospital Zurich where I was in Academia working on drug transporters, and with this knowledge on drug transporters I came to Roche and I worked on drug transporters at Roche in the lab at the beginning. Then I switched to biomarker research within Roche, which was on the backlab side looking into clinical trials and supporting clinical trials, doing a lot of sequencing at the time on Tarceva trials and so on. I switched to biomarkers more at the late stage phase in helping the teams to develop biomarker programs, to develop prophase 3 protocols and to bring these biomarkers into clinical trials from the exploratory side as well as if you have a predictive or prognostic biomarker, to the development side. The next step was now to go into the companion diagnostics and to develop assays and help to file assays and make assays valid and show the clinical validity of these assays, for these biomarkers that we find. So, I think this is a long way through this company, and you know, this is what is very fascinating. You work in teams, it's different from Academia, you work in large teams, but you have also a lot of influence on decisions. You can take decisions within these teams. But, you also have some freedom; if you have ideas, you can propose these ideas, but you have to go through many governance committees to raise money for it and to get money to really bring this into life. But it is possible.

Christian Schäfer Frank, one question to the science and entrepreneur guy: Those are all really nice guys. Could you as a company and academic institutions think of a project you guys all do together?

Frank Diehl Yes, absolutely because Industry has to focus as I mentioned, and there are some new clinical questions for oncologists the laboratory people would like to ask and Academia and we can try to facilitate that.

Christian Schäfer So, do you have meetings where you all sit together like Roche and your company to… [laughing]

Frank Diehl Yeah, Roche not so much [laughing]. No but the Genentech side which is interesting. We have done also work with early stage clinical trials with Genentech, so once they go into later stage, I mean there is an interest also.

Christian Schäfer Do you think, I always had the opinion the Genentech guys were like the Google guys, the cool guys, what is your opinion?

Frank Diehl I think, as you mentioned, I mean the company has been around for some time and the influence of a traditional pharmaceutical company has changed Genentech also in that respect, I don't know, I mean…

Christian Schäfer So, no clear "Yes" to a cooperation between all these companies and academics. Alain, maybe first one question to your newest start up. You didn't answer that question earlier, but maybe first the other question I had for Frank: Could you imagine talking to Roche and working with those guys and the Uni in Bonn?

Alain Thierry Sure, in fact, even as an Academia employer you can discuss with Roche because there are some RND-projects and when these RND-projects are producing intellectual property you have a combined interest. Sometimes there is a development of a product diagnostic and so in fact the frontier is not that far. The point is that when, as I mentioned, there is an intellectual property from a team, only from a team, an Academia team, you need to protect, so you produce a patent. This patent has to be valorized, so either you contact companies, a small company or a big company or you create your own company., this company can be swallowed by a big one, or develop an early one. For us, we knew that when you are entering the clinical setting you need a lot of money to produce a very interesting clinical trial. So a small company has to quickly develop to be very effective or to be associated with a big one. So there are different strategies and it depends also on the objectives of the company, the strategy. You can have a company with service, a company of RND or a company selling kits or a company selling drugs and compounds, so it's different objectives, different means and different ways to work.

Christian Schäfer Liquid Biopsy – I was really amazed because I was filming three weeks ago at Charité here in Berlin and it was my first contact with liquid biopsy in a prenatal situation. And so this was my entry and my briefing for today. A question to the audience: Is liquid biopsy ready for clinical practice? Yes? Please raise your hand, No? OK, neutral: Well, we can't really use that.

Christian Schäfer Ellen, is it ready? Because you have mentioned earlier that it is not, or you're not sure?

Ellen Heitzer If you mean, you can really implement it in the daily routine, I would say "no", but as we do it on kind of a research level to help patients that are, let's say end stage or late stage, and the clinicians want to do something – at least something. If we do our analysis and we can help them, at least for a few months, then I would say "yes", but to really implement it as a diagnostic test, I think there are so many open questions. What we have heard today, what is the actual source, what are the dynamics, what is the origin, and so on, does it reflect the driver clone or does it reflect passenger clones, and so on. There is so many open questions and I think these questions need to be answered before really offering it as a routine test.

Christian Schäfer Stefan, what is your opinion?

Stefan Holdenrieder I think a lot of work has been done on the methodological part and if I compare it with earlier CNAPS conferences, this is the first conference where we see really large studies dealing with liquid biopsy and this is really encouraging. It might be that it's not at the point of routine diagnostics yet, but I see that we are on a good way. There are some points that have to be fixed, this is pre-analytics, this is standardization, this is quality control, but then I think we also have to discuss where it can be placed and where it can be done in a high-throughput, high-quality manner – and then it's ready, maybe for the next CNAPS conference?

Ellen Heitzer And, sorry, I think we really need to do clinical studies, standardized clinical studies, with large sample sizes to validate its use as a clinical biomarker. And I think we are close, but not there yet.

Christian Schäfer So, looking at our team here on stage, I think we should hire Mark for acquisition of patients for studies because the figures are good.

Mark Eccleston Well, we've got the collaborations with the clinicians to provide the samples

for us, so we can't take credit for that. That's maybe through reflection on the belief in the technology in a way that we can promote ourselves, but you know, we absolutely need the clinical and academic collaborations in order to do that because without them we are nowhere,. But, I would say, actually Stefan was saying, it depends on the application. Ultimately, what you want to do with the test. So I agree absolutely with what you're saying, but I would also say we are planning on launching test next year, so…

Alain Thierry Yes, the panel here is more focused on oncology, and we have to mention that the work of Dennis Lo, and all the work on pregnant women. He said in his talk that one million women were tested, so what I believe is that, in the light of this domain of pregnancy and inheritable diseases, I think the work in oncology can go by the same way – and with the steady work of Dennis Lo we have to follow his way of working.

Christian Schäfer Michael…

Michael Fleischhacker (from audience) I just want to extend your question. I would really like to know from everyone sitting on this round table, where do you think will liquid biopsy be in 10 years? Is it a system, a method which can be used as a stand-alone system or is it just an addendum – or maybe – to the real biopsy? That is for me a question to which I would really like to get an answer.

Christian Schäfer Johannes…

Johannes Noe Yeah, maybe I can also give some thought about what you were asking. I think they are complimentary and they will stay complimentary. I think there are a lot of things we can learn from tissue, as well as we can get from liquid biopsy. Taking the example of lung cancer for example, which is very molecular diverse nowadays, and dissected into small pieces, we have sometimes very well-prevalenced biomarkers there and we have drugs developed against these well-prevalenced biomarkers. I think, especially in this disease, we don't have the luxury of having a lot of tissue, right? We cannot look at all these biomarkers, which have 1 % prevalence or even lower and in this scenario I see liquid biopsies, I think they will rapidly come up, and will be very important to really diagnose these patients and to see which of the personalized drugs or which of the target drugs will be the right ones for these patients. But, as well, I would say to put liquid biopsies into clinical practice, I think we still need a lot of prospective trials to run and to see how they are in relation to efficacy, how they pay out in relation to efficacy? What we find, I think that's very important. And the other point I wanted to make: we talk about liquid biopsies and we heard today it's not only one thing, right? It's CTCs, it's circulating DNA, it's circulating RNA, it's all kinds of different things, it's not only one thing. I think maybe the circulating DNA will make it faster than maybe other liquid biopsies.

Frank Diehl What I see is that the main striving force right now in oncology is the predictive value of the somatic mutations I think this is the main driver and we see commercial uptake, for example EGFR testing. There is a CE-IvD-kit, it's basically for routine use; there is no or little tissue available and there is a need for testing. The pressure is high, basically from pharmaceutical companies, to be able to stratify the patients. The other application for the long term is monitoring. I think there the competitive advantage of blood-based testing is very big; early detection is also a very big potential, but we also have to manage the expectations to the patients and investors because I see also today, there are some talks that are talking about early detection, but we have to be careful not to oversell this approach. I think this is very important.

Stefan Holdenrieder I'm very thankful for this and I would like to encourage the industry to follow this path because I think that monitoring is much harder to meet. We have to define exact time-points when we do the measurements, we have to define which changes are really relevant for changing the therapies and which methods we add afterwards as additional diagnostics tools.

And so I think this would be the next step we have to do in CNAPS also, do large studies on this topic because for all the other markers in clinical chemistry, for example, all these studies have not been performed and we have CEA and other tumor markers for 40/50 years now and we still do not know which changes are really relevant for therapy-monitoring, therapy-guiding. This is the chance to do it now and I hope we can do it now.

Christian Schäfer Just have Michael's question answered by everyone. Alain?

Alain Thierry I just wanted to know why Michael seemed to be so pessimistic?

Michael (from audience) I'm not really pessimistic, but to give you one example, I mean with DNA. I would say we are not yet ready, but we are getting there. With RNA it's a completely different issue, and whatever is published on RNA I don't believe that. If I would be a patient I would really not want that anybody suggests whatever treatment or not a treatment based on published data. So, therefore, I'm very cautious. DNA is making progress, and we are really on the way. I'm really pretty much sure, because we have a test system for therapy monitoring of lung cancer patients that was developed and certified by Epigenomics and it works very well. It's very robust and we generated really nice data That is according to our experience – solid ground, everything else, I wouldn't really go over that bridge.

Christian Schäfer Maybe just on Michael's question: liquid biopsy in 10 years?

Tobias Paprotka Of course I can't tell where we'll be in 10 years, I can just comment on a methods perspective. So if you imagine circulating DNA, it's probably the most difficult material for NGS-sequencing, so it's present in very tiny amounts, it's highly degraded, and if you think back like 5 or 6 years, it probably was not possible to really create high-diversity libraries from this material. Here, the protocols made real advancements and also very unexpected advancements, like desegmentation just came up. I think that nobody ever thought of this before, so I think there are some interesting methods coming up. I think nanopore sequencing is known to almost everybody here and possibly with these new methods, and also being able to sequence circulating DNA in a very high depth, so the diagnostics will change a lot. I'm pretty sure about that.

Christian Schäfer Ellen?

Ellen Heitzer I don't think that we can stop analysing tumor tissue. I agree, fully agree, in terms of RNA, in terms of histology and so on, but as for monitoring purposes I think the liquid biopsy is a better reflection of the overall genetic composition of the tumor, rather than a single biopsy of a metastasis because you don't know about heterogeneity in the biopsy and if you just have a needle biopsy of this clone and you won't get the other clone, then, yeah…. That's why I think that the liquid biopsy is the better tool for monitoring, but for diagnosis I'm not so sure.

Christian Schäfer Mark?

Mark Eccleston I'm very optimistic, I'm always optimistic. But I would say that I think the common area on the talk about stratifying rather than going straight into the final diagnosis, so the early warning systems and doing more conventional biomarkers, I'd say that's kind of Litian's approach that we would like to see within 10 years – that our type of technology – I wouldn't say ours specifically – but our type of technology could be replacing things like stool testing for colorectal cancer screening for example, those sorts of relatively low hanging fruits and maybe that will build some kind of credibility into the field for other people to follow on.

Christian Schäfer Are there challenges at the point of care?

Mark Eccleston I talk to the group at point of care as well, because that kind of helps with the

pre-analytics that everybody – it's kind of the elephants in the room almost, what do you do about it – and if you can remove as much of those issues, by doing a test at point of care, then that's gonna be beneficial and again it depends on the application. sSo the more sophisticated applications of sequencing and stuff like that, maybe the preparation and the requirements for the point of care test are more difficult. (…) It's relatively straightforward, you can get a pregnancy test, a lateral flow test – they exist already, so translating onto that platform maybe more straightforward.

Christian Schäfer So, I won't leave this stage tonight without a very concrete plan on how we can all work together. So how can we start this? Academia meets Industry, this is the topic for tonight, Ellen, how do you approach industry if you have an idea for a project? How do you do sales?

Ellen Heitzer Thanks God, my boss is doing this stuff [laughing].

Christian Schäfer Good answer.

Ellen Heitzer That's the truth, so, in most cases, Industry is approaching us. So for example we have a project with Janssen where we follow prostate cancer patients from castrations sensitive to resistant. They were approaching us, whether we want to do our analysis for them. So, that's not really a problem and we also in this EMI-consortium where also Alain is a partner and many more people. I don't know whether other guys are here in this consortium. This is also, or has the aim to establish, all these pre-analytical standard operating procedures, comparing result within-labs, between labs and so on. There are also several industry partners like Servier and other pharmaceutical companies and so it's not really a problem. I think, Academia already meets Industry; there are a lot of EU-projects that make this available, so I don't see a big problem of cooperation between Academia and Industry.

Christian Schäfer But let's not talk about problems, but challenges and plans how to get there. Frank, there must be topics, general topics, where you could even work together with Roche, like processes and so on. My question is, because I really love this meeting here, it's a very good atmosphere and you have workshops, but for next year for example I could imagine you have three more workshops where you talk between Academia and Industry about how to implement processes to work better together in the future. Do you have ideas for that?

Frank Diehl So from the academic, I mean what Industry I think is requiring from Academia is to have the concepts developed in Academia, to have the freedom to test different hypotheses, and to come up with new ideas. To participate in trials or to actually organize these trials, biomarker and technology IP. I think the main job of Academia is to drive this type of new innovation. And then Industry's goal should be to professionalize, to standardize and then the bigger the industry gets the more standardized, the more broader it is also to disseminate technology to every area. I think this is one of the things that makes Roche very valuable – to take these diagnostics globally. Not just in a service lab in Baltimore, but to globalize it and to take it to every country; there is the infrastructure in the big industry to do that. I think this is the two levels, that Academia, the ideas and then the large industry to penetrate global markets.

Christian Schäfer OK. Alain?

Alain Thierry I'd like to add one thing that link the industry and the Academia lab, is the fact that there is more and more transnational investigation linking the clinical area and the laboratories. And then from this association, the industry is more, by history, linked to the clinical area. tThere is a better link between the laboratory and Academia and the industry, because there is already association and studies and the clinical trials are made between the clinics and the industry. So, the labs are more close to the clinic, and the association is much better now I suppose.

Stefan Holdenrieder For us there are two interfaces. One interface is in Academia; you have some ideas and develop something and it works. Now you have a proof of principle and then you need the industry to develop real products, professionalize it and to bring it to a stage where it can be used for clinical diagnostics. Then it's a second interface where we have to early test these new assays in the clinic with real patient samples, to see whether pre-analytics, methods, and also the clinical validation, are really true. And, therefore, I think the close collaboration between Industry and Academia is necessary in two ways.

Mark Eccleston I agree with Stefan, I mean, in terms of what we're doing. We have ideas and plans, so we're not really actively looking for new technology, but on the validation side and the pre-analytics, and the CFC mark, the externalization studies, laboratory reproducibility, and also on the clinical sample side. As I said, we couldn't do what we're doing without clinical collaborators to provide those clinical samples.

Christian Schäfer And how important is a good database, a good patient database on the clinical side?

Mark Eccleston Actually that's hugely important and that's one of the big advantages of these that we're carrying out in Denmark, just the way the Danish system is set up with the patient data. Now the information goes all the way through. Some countries do that very well, some countries have chosen not to do it for a variety of reasons – people think that you're using my data, you know what's happening to me, and it's, you know… I don't understand that personally, but I can, yeah, that's fine, but I mean that's restrictive in terms of what you can do going forward, so… patient data is critical, completely anonymous, there is no need to personalize it, but it's hugely valuable.

Christian Schäfer So, liquid biopsy doesn't seem to be really ready for the market, for the mass market. What can we do together to promote this?

Mark Eccleston I come back to what I was saying, I think it's dependent on the application. So if you can simplify the applications, and you can see the utility and the clinical utility and the answers that you can provide, then I think to an extent it will sell itself. It's not just that you've got to convince the clinicians, who may be using the test, you've got to convince the reimbursement side of things. You might pay for the test, so it depends on the route how you go into the market and what you can do with it and the back end. You've got really critical decisions you're making on treatment, it's almost as a last result maybe, and it's almost a shame that it has to be like that.

Christian Schäfer You have a question?

Man in Audience Sorry, I didn't want to interrupt the conversation. I work as Michael. I work at Birmingham Women's Hospital, so you could say that I'm part of Academia, more or less, and I don't think I'm sure exactly what the aim of the collaboration between Industry and Academia is. I don't think it was actually pointed out very well. So, is the aim that we develop things faster, so they can get to the patient faster? I see many nodding. OK, in that case, my experience has been quite different with the Industry. I had different experiences, so I've seen like, "Oh yes, we can collaborate" and everything, but sometimes its difficult to speak to the Industry, when they make a couple of calculations and think, well, is this really worth it, in the sense that working with rare diseases, rare genetic disorders for example. It's quite obvious that you're trying to make something for a very small part of the population and I've had high and lows speaking with different companies where I have approached them and said "I want to do this, and it's not exactly how you've made the kit, maybe could you help me?". I've had some companies say, "Yes, we'll help you, get on with it", and some other companies said "No, sorry, we just don't do it this way" – and I never heard from them again. And this has happened a couple of times. aSometimes I ask questions "Where do you see this going?" like single gene disorders, and I've heard answers like, "Ah you know, single gene disorders are

very complex, you have to get all this done, very deep sequencing" Then they turn to "Let's talk about micro-lesions for example". So I just wanted to ask kind of a hard question: Where does Industry meet Academia, where there is not a clear benefit from the industry point of view? Thank you.

Christian Schäfer That's a tough one. I think this is for Mark.

Ellen Heitzer That's what I just wanted to say, the question is, as you said, what is the application, and the next question is, what is the benefit or the consequence, if I have the test, what is the consequence, do I do surgery or not? Does the patient have to get adjuvant therapy or not? Is the druggable target identified or not? And if so, who is going to pay for this? If I identify a druggable target, is the insurance company paying for the therapy change, is the pharmaceutical company paying for this, so that's all open questions.

Mark Eccleston I'm probably the wrong person to ask, because I like doing cool stuff, so if it sounds cool I'd probably want to do it, but from a commercial perspective obviously that's not necessarily the right way forward. I don't think you can necessarily predict what's going to be useful or what's going to be a good commercial product too far in advance. The company is there, it needs money to function. Academia can't survive without money. It get's that money through grants, so if you're in a popular area, exosomes for example, maybe there is lots of grands being given in exosomes. t's only the same for companies, you have to convince someone to give you that money, that could be your own money. In our case, when we started off, everybody put their own money in. It could be from the stock market, but at some point there is a pay back, so you have to convince your investors to stick with that.

Christian Schäfer And as we heard from Johannes, it's the same at Roche.

Johannes Noe Yes, it's the same. If you have a good idea it has to somehow meet the portfolio and the reason of the company What you want to achieve and where the projects are going, and I think you don't find the money on the street. Even in the company, you have to go through many governance committees and convince them with data, with whatever, maybe you get something to start a project, to do a feasibility, right? So a small amount of money and then you go back and say "We show you data now, this is working, can we bring this forward?" – and this is the way how we have to work as well. So we don't just get a pot of money and do with it whatever we want. And that's also I think in collaborations with Academia, if there are good ideas, and I think we can bring them forward, we can plan a project together and fund it to a certain extent, and this happens in reality, so…

Christian Schäfer But the moment you wake up with a great idea, you put it on your flip chart – and what do you do next in Roche?

Johannes Noe In principle, I normally discuss if I have an idea. I discuss it with my peers, I discuss it in my group and bring it forward, and maybe make a presentation in a broader group, and see how the reaction is and if people, you know, you see it fits, or if people are more reluctant or so, that's the way how you do it. You try to spread it and you try to discuss it with your peers and then if, also if you're within a project, you discuss it with your project team and if the project team supports you on this, then you can move on with such ideas. That's normally the way how I approach it.

Christian Schäfer And at which point do the health economics, the reimbursement guys come into the game?

Johannes Noe That's not so early, you can do feasibility first and if there are data, at a certain point there might be people coming into the game

and calculate the revenue in the end, but if you spend several millions you want to know how you get the money back, right? That's understandable. But this is not at the beginning.

Christian Schäfer Tobias, how is it in your company, if you have an idea, what's the process of innovation management, project management?

Tobias Paprotka Actually, of course the field is moving very fast, so very frequently new protocols, new methods, new technologies are coming up. So usually we start with discussions, evaluations, but I have to admit relatively fast the question is asked "How can we sell this? Is it possible somehow to sell it? Does it really make sense to put more efforts in that? How does it fit the market, is it really fitting in our portfolio?" So, there are many really great ideas, but only very a few really sellable for us, so we can really bring them to the market. It is always a very tough decision.

Christian Schäfer Do you have an exchange with Academia, like in a way that you invite scientists to spend half a year with you, programs like this?

Tobias Paprotka We are all scientists working there in our RND-department, so basically we are all coming from science and we have still connections to the university, and one of our major connections is through publicly funded projects where we work together with universities and also clinicians, and I think here we kind of align a bit.

Christian Schäfer Alain, you still haven't answered my question regarding your last start up. What are you doing with the new kit…

Alain Thierry Yes, in fact, I have the same story as Nitzan Rosenfeld, who also in the same time created and cofounded Inivata. So in fact, it starts from technology improvement, intellectual property, and then you have to, as I said before, you have to decide what you are doing with this intellectual property. So as an academia worker, I have to talk with the administration on what they are going to do and in fact, there is lot of freedom for the Academia worker to decide what to do. And in France, there is the huge advantage that there is a lot of facility for scientists to found a company. So, it's more or less easy. I think Nitzan will say the same maybe in the UK. So then what is important is that you have knowledge, and if you sell a license, in fact you sometimes you lose the knowledge of the intellectual property. So many times it is very important to combine the people who invented the story, to combine the collaborative project with the industry and then either to have RND-project or a creation of a biotech company. It is very difficult because you have to find investment, you have to decide if the founder will receive a lot of money or not a lot of money, because you can be deleted very quickly, so that's also a strategy of the founder. sSo if you are deleted and you don't have the hand of the company, or you can decide to have the hand of the company, by not being deleted and finding low investment. So there are different strategies in biotech and it depends also on the strategy, RND, kits and also services.

Christian Schäfer Frank, do you miss the start up days?

Frank Diehl Sometimes. This is to be flexible and to be fast. Actually I want to try to answer the question that was asked in the audience. So I had time to think about it. So I think the big advantage of working together, not only the IP and ideas, but also that Academia creates publications that are put out and that the company coworkers realize: "Oh, this is a new paper, what do you think about it" and it creates a discussion forum and it's an independent source of ideas. So that's why I really think it's great. There comes a Nature paper, so I immediately get an email, and say "What do you think about this new technology, blabla", so I think this is a way of generating excitement, also in the company and to get a support to go into a certain direction. If I just talk to myself and say "Oh crazy, go back to the lab",but if there is a Nature paper, or whatever paper, there is media sometimes covering it now: Liquid

Biopsy, you have seen many reports in the public, that's what creates excitement and also in the management of a company. So that's why it's good to have academic research.

Woman in the Audience I think (…) has proven a good idea (…) just a small population patients (…), but it's not commercially viable, because it's a small rare disease.

Frank Diehl Oh, these orphan diseases are problematic. Yeah, that's true, usually when you make your business case, you have to have large numbers and to justify the investment of an IvD-product. You have to heavily invest, there is the whole regulatory path, there is the studies you cannot do with the small sample numbers, there is no revenue, and I think the way it has been dealt with in the past is the LDTs, the laboratory developed tests. In the United States, that's the exemption, the clear guideline has is to do rare testing, this was the original idea of LDTs. Now, they are carved out because people are trying to get around the FDA, but in the end this is the way to do it, by laboratory-developed test. You have less investment, you have a shorter regulatory path, you don't have to run these large clinical trials. So I think this is the way the industry can adjust by offering this LDT. Not as a way to go around the regulation, but as a way to look for orphan disease.

Christian Schäfer Stefan, how do you pitch your ideas to industry? Do you write a presentation of 30 slides, do you write a letter, how do you approach industry?

Stefan Holdenrieder So, I'm in contact with the industry and I think meetings like this one here is one opportunity or other meetings and I think to be in constant exchange of thoughts is very important because then I hear the needs of the companies and I understand also that they need a benefit, but I also can say what is necessary from a doctor, a medical point of view, and that the quality is OK and that you consider all these influencing factors and that you have a good standardization and quality and that you have a good clinical validity. And to test this and to do this together with the Industry is very important because you cannot do it alone all the time. You can do it for one or two things, but then if you want to show that these new technologies are better or additive to the ones you already have, you need the industry. And, therefore, I think the continuous exchange of thoughts is very important.

Christian Schäfer And how do you teach your team how to approach industry? Is it always a team effort, or is it just you doing it?

Stefan Holdenrieder I think the beginning is often done by me, by myself. Then, in the process of being in contact, there are also contacts on other levels, at the working levels, doing the tests themselves. Then the contacts are there on various levels and then I also get a feedback from my coworkers, what has to be done, or what we have to talk about and then it creates a network of exchange.

Mark Eccleston I just support what Stefan said, that in my own personal experience when I started my PhD, my PhD supervisor was very well-integrated into the commercial environment and we were always meeting people. I was part-funded by the industry for my PhD, so those sorts of levels and then that's kind of engrained into me now. So when you meet principle investigators from academia, then you always talk to their post docs and then, post them maybe, get them to work for you, that level of interaction feeds down and in sort of cycles.

Christian Schäfer I think it would be a good topic for a workshop, how to pitch ideas to industry. I mean maybe a start. Mark, what's the most challenging task on your to do list on Monday?

Mark Eccleston I have a conference call with Horizon 2020, consulting, trying to set up an application for a grant, so that actually is quite challenging for the next round. This is exactly the sort of thing, so there is gonna be "it's an SME-call"; so we will be leading the call, but we will be interacting with clinic partners on it.

Christian Schäfer Ellen? Most challenging, the one you might not be able to sleep tonight…?

Ellen Heitzer We've just submitted a paper and we got the reviews back. One review liked us very much, the other did not and we're going to submit it to another journal on Monday, so, I have to go through the paper.

Christian Schäfer Tobias?

Tobias Paprotka I'm actually off on Monday, so my task will be to fly home around midnight. That might be a bit difficult! But no, I think to do some calculations, nothing special…

Christian Schäfer Stefan?

Stefan Holdenrieder I do not know, I will see on Monday.

Christian Schäfer Johannes?

Johannes Noe It's work as usual, I think. There is nothing special going, on, so pushing on in the project is the task. You have to deliver, right?

Christian Schäfer Seems pretty relaxed, on the academia and the industry side. Frank?

Frank Diehl What I'm trying to do is to get alignment, internal alignment in the company. The communication is important, so I'm trying to communicate to all the stakeholders to make sure we are all on the same page.

Alain Thierry My own challenge is to combine my role of academia worker of my group and to help on the biotech company we created. Also to have an eye on the fundamental area, because of the fundamental area, for example biophysical studies examination are very important to produce new idea and new technology. And sometimes, it's very difficult to keep the time on work and investigation. So you always have to be strong on the fundamental area.

Christian Schäfer Any more questions from the audience?

Man from Audience (from a biotech company) [introduces himself] I have another view of this, because early on we talked about where will be liquid biopsy in 10 years and we heard a lot about exchanging ideas and so on, but where academia and industry should really go together is education. We have seen this yesterday. If we want to make liquid biopsy really successful in a broad routine use, we have to educate. This means, here the CNAPS conference is a quite familiar club, but you have to go out, you have to educate the physicians, you have to educate patients, you have to create a market for liquid biopsy and then I'm pretty sure it will be successful.

Christian Schäfer Do you think so, Frank?

Frank Diehl Yes, very good. You heard this in the prenatal field, how important it is and I think the same will be true for oncology.

Ellen Heitzer I fully agree actually, but I think there is already a market, because a lot oncologists are familiar with the liquid biopsy and it's more and more getting into the oncology conferences. So, I think there is a market, but I fully agree that the oncologists have to be educated in terms of what is the result of such a test. So, it's not quite easy, let's say it's easy to report the fetus has a trisomy 21. That's a yes or no answer, but in the oncology field the question is "is this indeed a druggable target"? "Is the amplitude of this focal amplification high enough, so it can justify a therapy change?" – and all these things. Another issue I wanted to add is all the mutations we are looking at, does anybody confirm whether they are somatic or in the germ-line? For example, we had three cases where we identified BSE2- and a BSE1 mutation. They turned out to be germ-line mutations. And this has then implications, not only for the patient, that has cancer, but also for the rest of the family. So who is going to check on these things and who is going to report, or council, the patient? What is the implication of the test result?

Stefan Holdenrieder So, I think it could be a chance here, we have heard a lot of different approaches for liquid biopsy and we have the industry here producing all these products. Then we will meet in 2 years again and have the new results, but it could be a chance to make a forum somehow that we exchange the results in-between that time, or help us together to make these big, large monitoring studies that are necessary. Maybe we can join the samples of the patients from here and there, then compare the one method with the other method and see how they correlate or not, or make some point for standardization, or external quality assessment or so. Maybe we can find a point in this meeting with you altogether where we can just put the ideas together and then distribute them again and that we can make it more vivid.

Alain Thierry Yes, and also we have to be open to the other fields because the oncology field has to speak with the pregnancy field, with transplant field. For example transplant, there is a new company which leveraged ten million dollars on the transplant field and they are testing five thousand people, What is unexpected is that they work with it on pre-implantation embryos, so we could determine the gene mutation on an embryo of three days. And it is very important that instead of removing some cells you can just detect free DNA in the embryo culture. And you can have the test. We could also, this way, demonstrate by quantitative-analysis that you can determine the quality of the embryo, because normally when you do pre-implantation of embryos, you have twenty embryos. You have to decide by eye, so the physician is just deciding, oh, this one is nice. With cell-free DNA we could detect the best embryo you can implant in women. So it is completely unexpected and I am in the oncology field and yet I could work on that. I think that this kind of conference can help to promote the discussion between fields.

Frank Diehl Actually, I had a great idea of education and I think, that Michael, you started that many years ago. You had a website which you – is it still alive? You know, back then when you did it, I thought it was a great idea to have an independent reference of all the liquid biopsy field. I think this could be a way to educate given that we have an independent organization. Coming back to the goal of CNAPS and how we are organized, so if CNAPS or this community is able to build an organization, to build a website that could collect this information for the public, for physicians, for whoever is interested, for scientists, this could be a way to educate. The companies have to do their own education by the marketing, but also to have an independent way to educate would be good. Just an idea.

Christian Schäfer Very good.

Woman from the Audience Another question, I would like to ask the experts round here: Just in case patient monitoring based on liquid biopsy will go into routine, who in your opinion will do the testing, the practitioner, the oncologist, the hospital, central lab, the pathologist, the MDX service providers? I'm curious to learn your opinion.

Ellen Heitzer I was asking the same question. You couldn't know and actually I have no answer. I have no answer yet who is going to do the test, and who is going to report the result.

Stefan Holdenrieder So, I would have a suggestion. This is not a definitive answer, but a suggestion we have in Germany to this case because for the monitoring you will follow a defined marker with the time, and then, if a recurrence appears again, then it's a question whether you do a more sophisticated analysis., The question was also because in Germany, pathologists try to get this technique and because it's liquid biopsy, but it's not really a biopsy because in biopsy you have many more features like necrosis, hypoxia, vascularization, all these things that are around the tumor or make the characteristics of a tumor. Here we have the molecular characteristics of the tumor, but the characteristics of a tumor in the blood we measure already for years in the laboratory. We have protein markers, we have microRNA markers or epigenetic markers or genetic markers, so these are different classes

and everything is in the blood. Our suggestion in Germany was to emphasize more on the profiling, that this is a liquid profiling rather than a liquid biopsy, but this is only a wording. You have two points that are important: one point is all these pre-analytical things and quality assurance things with the blood and we in the laboratory are equipped for this. The next point is that you have to have a high throughput line because if you do not only do this measurement for single patients, but in the monitoring, this will be many, many determinations and so if the industry would be willing to do this in the central lab, this would fit very well into the diagnostic strategy we have at the moment. So this is an offer to put it in the central lab, because in Germany all the blood diagnostics are done there. How the reimbursement is via the oncologists or other things, this is another thing and if there is a more sophisticated analysis, that you do whole genome sequencing or so, I think this could also be a point for the geneticists, if you do the tissue diagnostics and of course the pathologists. This I think would, in the end, make sense if we have the diagnostic centers where all these specialities work together.

Ellen Heitzer That's at least what we are doing in Graz, so tissue stays at the pathology, blood goes to human genetics. But I don't know how this is in other countries. In Austria, a genetic test has to be performed by academia, or university institute. We don't have all these private laboratories in Austria.

Stefan Holdenrieder This is a central laboratory in the universities, as well…

Mark Eccleston So, I know in France they centralized laboratories, We went to visit one in Paris as part of our market research to see how we could implement our tests and where we would sell them and who we would sell them to. Going back to the example of screening for colorectal cancer, they screen 16.000 samples a day – that's what they are building up to, so it's a huge amount and the throughput required to do that level of testing is significant. In the UK, it's more fragmented. There are individual screening centers, distributed, but established specifically for that application. You have to ask the question, from industry, you have to ask the question where are we going to sell our products. so it's interesting that it's coming from the other side as well.

Christian Schäfer Any more questions from the panelists to the panelists or from the audience to the panelists? Otherwise I would like to thank you, thanks to my panelists, thanks to the audience and have a very nice evening at the Ramba Zamba, I'm looking forward to that…

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P.B. Gahan et al. (eds.), *Circulating Nucleic Acids in Serum and Plasma – CNAPS IX*, Advances in Experimental Medicine and Biology 924, DOI 10.1007/978-3-319-42044-8