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Effect of DNA target size on the efficiency of chimerism measurement in circulating free plasma DNA

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Abstract

Introduction. *The analysis of free circulating DNA (cfDNA) holds promise for molecular diagnostics, but its fragmentation and low concentration can complicate PCR analysis.*

Objective. *To investigate the effect of target length on the amplification efficiency of Y-chromosome markers from cfDNA.*

Material and methods. *Fifty cfDNA samples were obtained from 39 patients: patients after liver transplantation (n=19), patients with acute leukemia after allogeneic hematopoietic stem cell transplantation (n=10), and pregnant women (n=10). In addition, we prepared 16 chimeric samples by sequential dilution of male cfDNA into female cfDNA from healthy donors. We determined the proportion of male cfDNA using the Y-chromosome marker S02, which is 211 bp in length as suggested by M. Alizadeh et al. We also modified Alizadeh's primer design to obtain a DNA target with a length of 138 bp. The proportion of male cfDNA was also determined by fragment analysis using the amelogenin Y marker (84 bp) from the COrDIS Plus kit (Gordiz LLC, Russia).*

Results. *In the three groups of patients, amplification of male cfDNA was more efficient when shorter DNA targets were used ($p<0.05$). In artificially created 'chimeras' with a known ratio of male to female cfDNA, analysis of a marker of 84 bp in length gave values closest to the real ones.*

Conclusions. *In the quantitative models tested so far, shorter PCR targets are preferred for the analysis of cfDNA.*

Keywords: free circulating DNA, target DNA, chimerism, liver transplantation, allogeneic hematopoietic stem cell transplantation, pregnancy

Conflict of interest The authors declare no conflict of interest.

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aGVHD, acute graft-versus-host disease
AL, acute leukemia
ALL, acute lymphoblastic leukemia

allo-HSCT, allogeneic hematopoietic stem cell transplantation
AML, acute myeloid leukemia
AR, acute rejection
bp, a base pair *(two nitrogen-containing bases (or nucleotides) that pair together to form the structure of DNA)*
cfDNA, circulating free DNA (also known as cell-free DNA)
CMML, chronic myelomonocytic leukemia
dcfDNA, donor circulating free DNA
DLBCL diffuse large B-cell lymphoma
FA, fragment analysis
fcfDNA, fetal circulating free DNA
FF, fetal fraction
GVHD, graft versus host disease
MM, multiple myeloma
NGS, next generation sequencing
NIPT, non-invasive prenatal testing
PCR, polymerase chain reaction
PMBCL, primary mediastinal (thymic) B-cell lymphoma
SNP, single nucleotide polymorphism

Introduction

Analysis of circulating free DNA (cfDNA) is a promising method of molecular diagnostics. It has acquired particular importance for several reasons. Currently, the prognostic value of donor/recipient cfDNA fractions in blood plasma in organ and bone marrow transplantation is being actively studied. In addition, more sensitive approaches are required to determine the proportion of tumor cfDNA in patients with cancer, including oncohematological patients. Determination of molecular markers in fetal cfDNA has long been used in prenatal screening.

With the development of transplantology, there is a need for non-invasive diagnostic methods to predict graft failure. Researchers are looking for new biomarkers to detect a rejection, asymptomatic graft damage, and immunosuppression failure. One potential marker is donor cfDNA (dcfDNA), which can be detected in blood and other biological fluids. This can help identify complications associated with graft rejection

and infectious processes at an early stage [1]. To date, many clinical studies have been published that convincingly demonstrate the possibility of using dcfDNA to monitor graft status [2, 3]. An increase in its fraction in blood plasma is usually associated with an impaired graft function, including a rejection. The dcfDNA has been studied most actively in organ transplantation as a non-invasive and accurate biomarker for monitoring liver graft status.

Allogeneic hematopoietic cell transplantation (allo-HSCT) provides an effective treatment of neoplasia and non-neoplastic diseases of hematopoietic tissue. Monitoring of post-transplant complications is extremely important, but current diagnostic capabilities are limited. The ratio of donor to host cfDNA fragments in blood plasma is used as a universal parameter for monitoring the most important complications that occur after allogeneic HSCT: a relapse of malignant neoplasm and a graft rejection [4]. To detect the graft rejection, the proportion of donor and recipient cfDNA is measured in plasma samples [5]. To confirm the relapse, an analysis for the presence of tumor-specific genomic damages in cfDNA is made in parallel [6].

The main causes of perinatal mortality and childhood disability are chromosomal abnormalities such as aneuploidy of chromosomes 21, 18 and 13. Aneuploid pregnancy can lead to occasional miscarriages, frequent bleeding and premature birth [7]. Ultrasound examination, determining the markers in maternal blood serum and computer analysis are the main methods used for screening pregnant women. These methods help to identify women with a high risk of the above pregnancy complications who require an invasive examination [8]. Invasive procedures such as chorionic villus sampling and amniocentesis are used to obtain samples for cytogenetic testing; cordocentesis is used less frequently. These procedures require careful consideration of

contraindications due to the potential risk of complications, including spontaneous abortion, which occurs in 1–2% of cases. After the DNA Y-chromosome circulating in the maternal bloodstream [9] had been discovered in 1997, noninvasive prenatal testing (NIPT) began to be developed and actively implemented. Numerous clinical studies have shown that the amount of fetal cell-free DNA (fcfDNA) is the most important parameter for NIPT, since its insufficient amount can lead to false negative results [10, 11]. Malignant neoplasms, maternal and placental mosaicism can also cause discrepancies between the fetal karyotype and NIPT results [11, 12]. Next-generation sequencing (NGS) can both determine the proportion of fetal cfDNA, and also genetically identify the sample. In addition to prenatal diagnosis, this approach can be used for non-invasive paternity testing.

For all these studies, it is necessary to statistically significantly estimate the proportion of fetal, donor, host, and tumor cfDNA that is present in blood in a fragmented form. The size of these fragments usually varies from 70 to 200 nucleotide pairs, or base pairs (bp), although in malignant diseases fragments up to several thousand nucleotide pairs long are found [13]. The main mechanism of cfDNA fragmentation is nuclease cleavage of DNA in areas not protected by nucleosomes. Circulating free DNA is mainly released from normal and tumor cells during apoptosis or necrosis (a passive release) and during active cellular secretion of extracellular vesicles and exosomes (an active release). Getting into the intercellular fluid, nucleic acids freely circulate in blood plasma and other body fluids: saliva, cerebrospinal fluid, and urine. The mean half-life of cfDNA in blood is 10–15 min [14]. The cfDNA is eliminated from the body via the liver and kidneys. In healthy individuals, the cfDNA level is usually low and averages about 5 ng/mL of blood plasma. This value may increase in response to physical activity,

inflammation, trauma, or other physiological and pathological effects. The concentration of cfDNA is considered a non-specific marker and may indicate the presence of various diseases, including oncological, infectious, and those associated with organ transplant pathology.

The aim of the study was to investigate the efficiency of detecting a target (donor, fetal) cfDNA depending on the length of the target DNA for PCR amplification.

Material and methods

The study included 50 cfDNA samples from 39 patients. Three groups of patients were selected for the study: patients who underwent liver transplantation at the Moscow Regional Research and Clinical Institute n.a. M.F. Vladimirskiy and were followed-up for 6 months to 13 years; patients with acute leukemia (AL) who received allo-HSCT at the National Medical Research Center of Hematology and were followed-up for 1 to 50 months; pregnant patients and employees of the National Medical Research Center of Hematology who were in the first to third trimesters of pregnancy or had lately given birth.

The first group consisted of 20 cfDNA samples from 19 patients who underwent liver transplantation between 0.5 and 13 years before the study start (Table 1).

Table 1. Patient characteristics of patients after liver transplantation

Sample No.	Patient code in the study	Patient's gender	Donor gender	Age	Period after liver transplantation, months	Plasma concentration of cfDNA, ng/mL
1	9 Myk	f	m	68	60	8
2	1 Dmi	f	m	48	23	11
3	36 Leo	f	m	52	6	27
4	35 Ger	f	m	46	59	30
5	12 Fel	f	m	59	130	10
6	15 Do	f	m	43	48	3
7	24 Bar	f	m	61	161	17

8	16 Skr	f	m	60	103	2
9	3 Kal	f	m	48	68	7
10	10 Kob	f	m	51	102	3
11	41 Bat	f	m	42	106	70
12	42 Kom	f	m	62	41	48
13	45 Kuz	f	m	47	105	23
14	11 TerA	m	f	58	108	5
15	14 Pav	m	f	72	127	23
16	37 Gav*	m	m	62	121	32
17	6 Mic	m	f	51	37	5
18	4 Gas	m	m	51	50	8
19	58 Pas	m	m	49	87	10
20	59 Gav*	m	m	62	133	13

Notes: * the same patient followed-up after 1 year; numeric designations in patient codes are arbitrary; cfDNA, circulating free DNA.

The second group consisted of 16 samples collected 1–50 months after allo-HSCT in 10 patients with AL (Table 2).

Table 2. Characteristics of acute leukemia patients after allogeneic hematopoietic cell transplantation

Sample No.	Patient code in the study	Gender	Age	Diagnosis	Donor gender	Post-allo-HSCT period, months	Plasma concentration of cfDNA, ng/mL
1	Gra*	m	35	ALL	f	50	35
2	Gra*	m	35	ALL	f	50	33
3	Gra*	m	35	ALL	f	50	37
4	174–999 Mol**	m	55	ALL	f	3	63
5	171–708 Gog	m	20	AML	f	2	75
6	174–206 Ana***	m	64	AML	f	1	30
7	1734–760 Mol**	m	55	ALL	f	1	260
8	174–561 Vas****	m	20	AML	f	3	40
9	173–534 Che	m	48	AML	f	49	27
10	175–799 Izm	m	36	AML	f	1	90
11	175–887 Ana***	m	64	AML	f	2	30
12	172–865 VAS****	m	20	AML	f	2	85
13	1512 Gal	f	28	AML	m	5	57
14	174–817 Iva*****	f	57	AML	m	6	21
15	174–705 Kar	f	23	AML	m	2	33
16	173–606 Iva*****	f	57	AML	m	3	33

Notes: *, **, ***, **** and ***** denote patients whose samples were tested more than once; numeric designations in patient codes are arbitrary; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; cfDNA, circulating free DNA

Circulating free DNA samples from 10 pregnant women, 4 of whom were healthy donors and the remaining 6 had various diagnoses including MM (multiple myeloma), AML (acute myeloid leukemia), PMBCL (primary mediastinal (thymic) B-cell lymphoma), DLBCL (diffuse large B-cell lymphoma) and CMML (chronic myelomonocytic leukemia). Details are presented in Table 3.

Table 3. Characteristics of pregnant women in the first study group ranked by the amount of male cfDNA in the fetus

Sample No.	Patient code in the study	Age, years	Status (donor/patient, diagnosis)	Pregnancy period	Child gender	Plasma concentration of cfDNA, ng/mL
1	151-269 Sve	36	donor	12 weeks	f	5
2	150-833 Xas*	41	MM	8 months after delivery	m	11
3	160-866 Sol**	37	donor	3 months after delivery	m	5
4	166-576 Gla	34	donor	10 weeks	f	6
5	172-939 Shm	37	PMBCL	36 weeks	f	53
6	151-853 Vor	40	AML	27 weeks	f	20
7	154-517 Sol**	37	donor	33-34 weeks	m	19
8	154-197 Xas*	41	MM	10 months after delivery	m	10
9	155-958 Bet	34	PMBCL	35-36 weeks	m	8
10	159-783 Erm	35	DLBCL	27 weeks	m	23
11	154-100 Shp	39	CMML	24-25 weeks	m	27
12	149-89 Sol**	37	donor	9 weeks	m	5
13	151-200 Ler	26	donor	22 weeks	m	8
14	141-633 Xas*	41	MM	38 weeks	m	18

Notes: *, ** patients from whom samples were taken three times at different stages of pregnancy and after delivery; numeric designations in patient codes are arbitrary; AML, acute myeloid leukemia; MM, multiple myeloma, DLBCL diffuse large B-cell lymphoma; PMBCL, primary mediastinal (thymic) B-cell lymphoma, CMML, chronic myelomonocytic leukemia; cfDNA, circulating free DNA

In addition, two series of artificial “chimeric” cfDNA samples with different relative abundances of male cfDNA were generated. Two pairs of 7 ng/mL cfDNA samples were collected from two healthy male and two female donors and serial dilutions of male cfDNA in female cfDNA were prepared. Samples with dilutions of 1/3, 1/9, 1/27, 1/81, 1/243 and

1/729 and undiluted cfDNA controls were also analyzed using three methods to determine the amount of cfDNA with Y-chromosome markers.

Blood samples (8–10 ml) were collected in tubes containing ethylenediaminetetraacetic acid. Samples were processed no later than 2 h after collection. Plasma was obtained from whole blood by centrifugation three times: at 800 g for 15 min for the first time; at 2500 g for 20 min for the second time; and at maximum speed for 15 min to remove cellular debris at the third time [15, 16].

The cfDNA was isolated from 1.5 ml of blood plasma sample using the commercial kit “CF Extra” (Raissol Bio, Russia), the procedure being performed in accordance with the manufacturer's protocol. The method for isolating nucleic acids from cfDNA is generally based on concentrating small DNA fragments with magnetic particles followed by purification. The resulting cfDNA was dissolved in 30–50 μL elution buffer “CF Extra” (Raissol Bio, Russia). We used the method of obtaining cfDNA and control genomic DNA (reference cell samples) from one test tube. The DNA from the blood cell sediment was extracted by the salt out method [17].

The concentration of the extracted cfDNA was measured using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, USA) with a kit for quantitation of samples with low DNA content (Raissol Bio, Russia).

The relative abundances of male DNA containing the Y-chromosome marker were estimated for all groups. In real-time PCR, primers and probes detecting the S02 marker on the Y-chromosome were used as recommended by M. Alizadeh et al. [18]. When studying genomic DNA, this test system allows the detection of minor DNA with an accuracy of 0.03%; the length of the target DNA is 211 bp. The *GAPDH* gene was used for normalization; the primers and probes used

were also as recommended by M. Alizadeh et al. (the target DNA length was 188 bp). In addition, we modified the forward (non-specific) primer from the Alizadeh kit to make the amplification target shorter (138 bp). The primers and sequences of the amplified fragments are shown in Fig. 1.

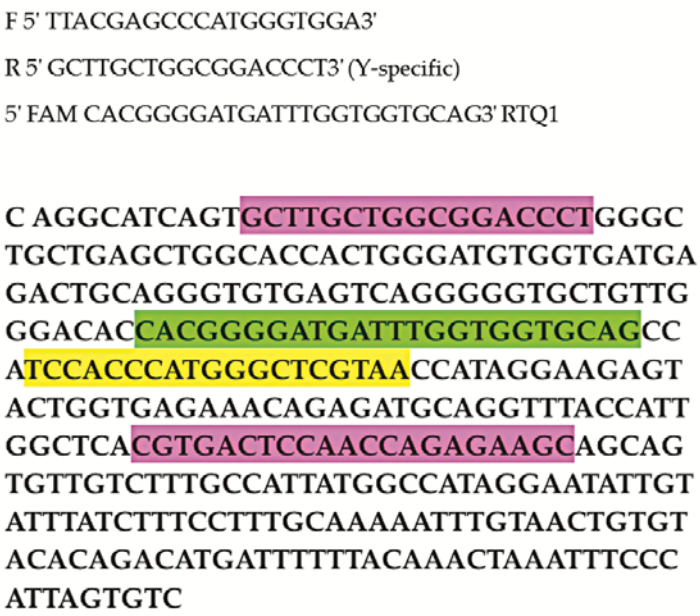

Fig. 1. Scheme of primer and probe arrangements to determine the DNA target of Y-chromosome in classical and modified test systems.

Pink color designates the forward and reverse primers (DNA target with a length, 211 bp) proposed by M. Alizadeh et al.; green color designates fluorescent probe; yellow color designates a new forward primer (DNA target, 138 bp)

The relative amount of male cfDNA was calculated using the ΔΔCt method. The formula used for the calculation was as follows:

$$QU/QC = (1 + E) – (\Delta CtU – \Delta CtC),$$

where QU is the amount of target DNA sequence in the unknown sample; QC is the amount of target DNA sequence in the calibrator sample; ΔCtU is ΔCt in the unknown sample; ΔCtC is ΔCt in the

calibrator sample; E is the PCR efficiency of target DNA sequence calculated as described by M. Alizadeh et al. [18].

In parallel, each sample was analyzed by fragment analysis (FA) to estimate the proportion of male cfDNA. We used the amelogenin Y marker (target DNA length 84 bp) from the COrDIS Plus kit to determine patient's STR profile (Gordiz LLC, Russia). The amelogenin X marker (80 bp) from the same kit was used as a calibrator. FA of PCR products was performed on the Nanofor-05 genetic analyzer (SYNTOL, Russia). STR profile analysis was performed using the GeneMapper v.4 software (Applied Biosystems, USA). The relative proportion was calculated as

$$Y = hY / (hY + hX),$$

where hY and hX are the heights of the Y (amplicon length 84 bp) and X (amplicon length 80 bp) peaks, respectively. In all cases, 5 μL of cfDNA solution with a concentration of at least 0.2 ng/μL were taken for PCR.

The results obtained were presented as M±SD (M is the mean value, SD is the standard deviation). To compare the relative effectiveness of different methods, Student's *t*-test for related populations was used (https://medstatistic.ru/calculators/calcpars.html). Differences were considered statistically significant at a statistical significance value of $p<0.05$.

Results

The diagram in Fig. 2 shows that the concentration of cfDNA in the three patient groups was high enough to accurately measure male cfDNA in these samples. In particular, cfDNA concentrations of 2 to 70 ng/mL in liver transplant patients and from 5 to 53 ng/mL in pregnant women allowed the detection of minor amounts of cfDNA starting from 1%. The cfDNA concentration of 21 to 90 ng/mL (in one sample it was 260 ng

/mL) in patients with acute leukemia after allo-HSCT suggests even more accurate detection of minor cfDNA and the possibility of identifying both the donor and recipient markers, and also the tumor markers.

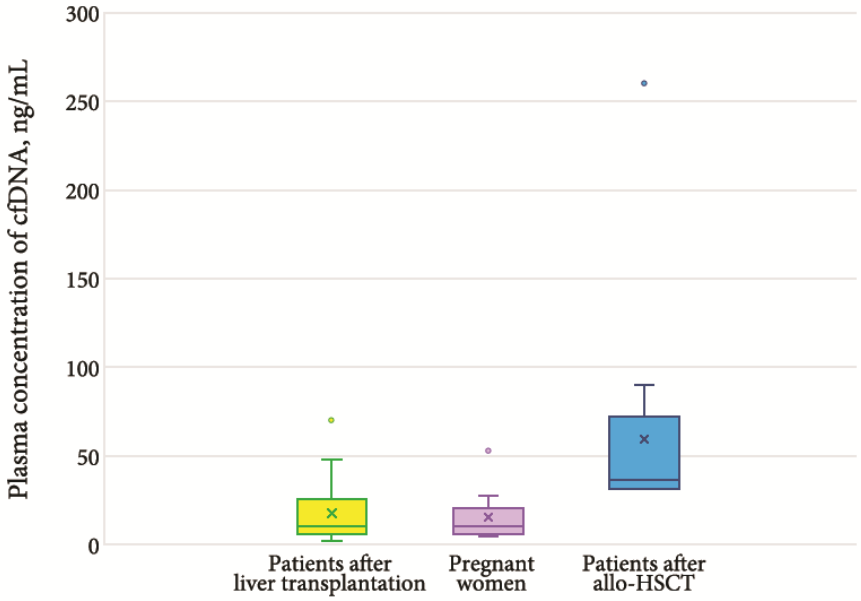

Fig. 2. Diagram of cfDNA concentrations in the studied patient groups

Table 1 demonstrated that, 14 of 19 liver transplant patients were women with a male donor, three were men with a female donor, and three were men with a male donor. As shown in Fig. 3, this gave us the opportunity to obtain a wide range distribution of the relative abundances of male cfDNA. It should be noted that patients 14 (female, male donor) and 15 (male, female donor) were at risk of graft rejection at the time of sample collection. In the remaining patients, the graft condition was stable, and the proportion of donor cfDNA did not exceed 25% according to the amelogenin Y FA. Comparing the three analysis methods, we found that the mean proportion of male cfDNA was 0.035±0.079 when using the standard PCR system, 0.096±0.150 when using the modified PCR system, and 0.350±0.388 when FA was performed using short amelogenin Y and X

targets. Student's *t*-test showed a statistically significant difference in the results between the first and second methods (t=2.919, p=0.009), and between the second and third methods (t=4.135, p=0.001).

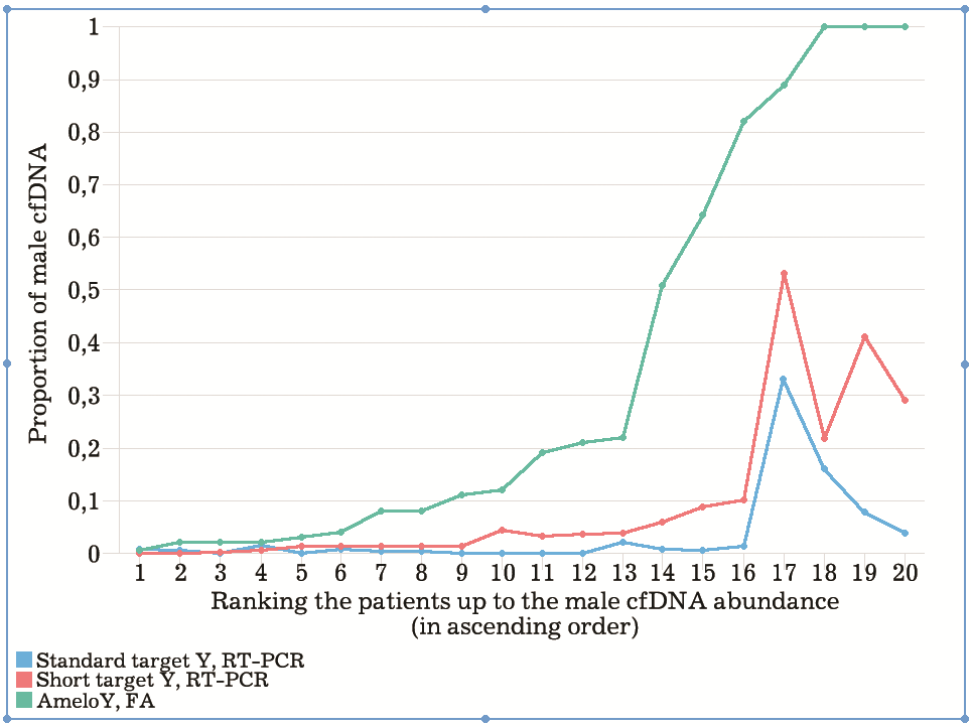

Fig. 3. Distribution of male cfDNA relative abundances in the patients after liver transplantation. RT-PCR, real-time polymerase chain reaction; cfDNA, circulating free DNA; ameloY, amelogenin Y chromosome; FA, fragment analysis

Similar results were obtained in the group of patients after allo-HSCT. Table 2 demonstrates that 12 of 16 cfDNA samples were obtained from male recipients who had received grafts from female donors and 4 cfDNA samples were obtained from female recipients who had received grafts from male donors. The highest proportion of male cfDNA was found in this group. The quantitative values of the male cfDNA proportion are presented as graphs in Fig. 4. Having compared the results obtained using the standard and modified PCR systems, we found that the mean male origin cfDNA proportion was 0.032±0.043 when using the standard PCR system and 0.150±0.177 when using the modified PCR

system. This difference was statistically significant (p=0.005) when Student's t-test was used. Thus, changing only one parameter – the length of the target DNA – while maintaining all other parameters unchanged, including the DNA normalization method, led to an increase in the detectable proportion of target DNA by almost 5 times: from 0.032±0.043 to 0.150±0.177 (Fig. 4).

Further comparison of the data obtained using the real-time PCR (RT PCR) and the results of FA of short targets of amelogenin Y and control amelogenin X showed that when analyzing short DNA targets, the proportion of the Y-chromosome marker was determined 2-fold more efficiently than when using the modified primer system for RT-PCR. The mean proportion of male cfDNA in the same samples was 0.341±0.308 versus 0.150±0.177, and the paired Student's t-test showed a statistically significant difference (p<0.001).

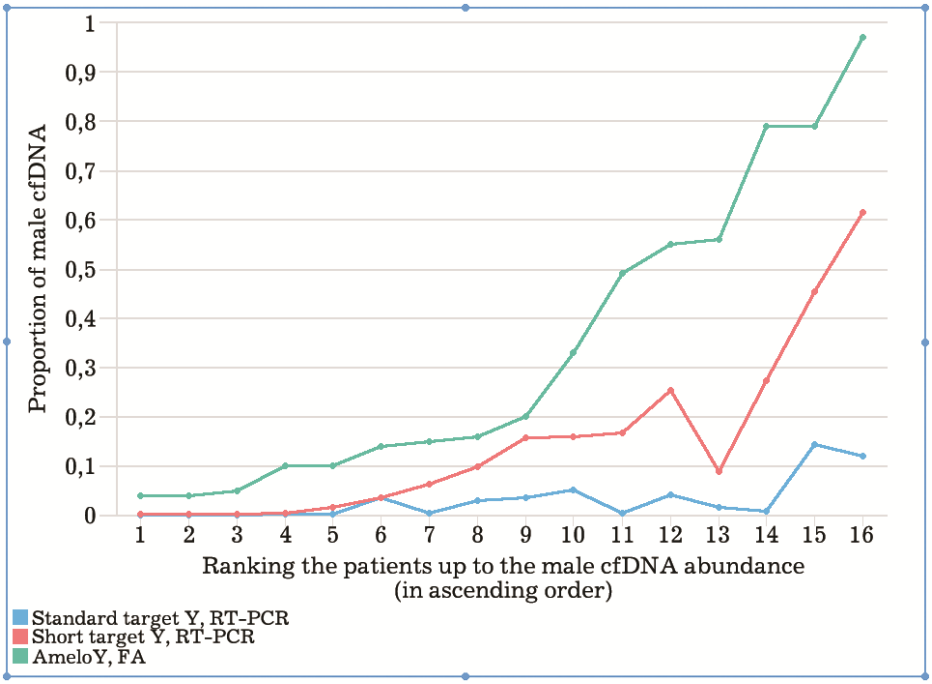

Fig. 4. Distribution of male cfDNA relative abundances in the patients after allogeneic hematopoietic stem cell transplantation.

RT-PCR, real-time polymerase chain reaction; cfDNA, circulating free DNA; ameloY, amelogenin Y chromosome; FA, fragment analysis

Fig. 5 shows the results obtained in a group of pregnant women, including employees and patients of the National Medical Research Center of Hematology.

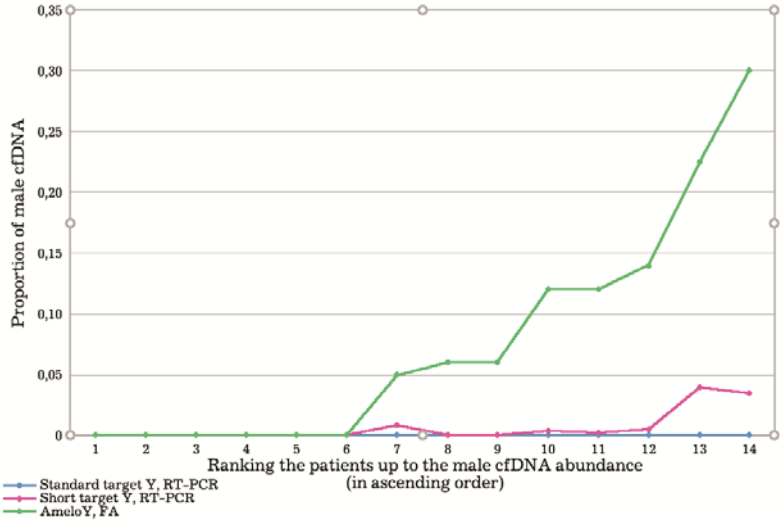

Fig. 5. Distribution of male cfDNA relative amount in pregnant women. RT-PCR, real-time polymerase chain reaction; cfDNA, free circulating DNA; ameloY, amelogenin Y chromosome; FA, fragment analysis

Compared with the transplant patients described above, the proportion of male fscDNA in pregnant women was significantly lower. And besides, this group included 4 cases where the fetus was female and 3 samples from women who had previously given birth to boys, as shown in Table 3. Therefore, the sensitivity requirements for assessing these cases were much stricter. When the standard RT-PCR test system was used, the Y-chromosome DNA-target was not detected in any sample. However, using the modified test system, it was detected at a very low level in 6 samples. FA of amelogenin Y short target and reference amelogenin X showed high sensitivity. In addition, male fcfDNA was detected in all patients expecting a boy, and in one patient with multiple myeloma; the amelogenin Y marker was unexpectedly re-detected 10 months after cesarean section. The mean proportion of male fetal cfDNA

was not detected by RT-PCR testing with unmodified primers and made 0.007±0.013 and 0.077±0.095 when studied by two other methods. The paired Student's t-test showed a value of 1.929, which indicated that the changes in the variable were not statistically significant when comparing between the first and second methods (p=0.076), but were statistically significant when comparing between the second and third methods (t-test value=2.834, p=0.014).

We made two series of dilutions of samples from two pairs of healthy donors – a man and a woman – and tested Y-chromosome markers similarly to the analysis performed on the patient groups described above. The electropherograms of the fragment analysis of PCR products and the amplification curves of target DNA in RT-PCR in serial dilutions of samples are presented in Fig. 6.

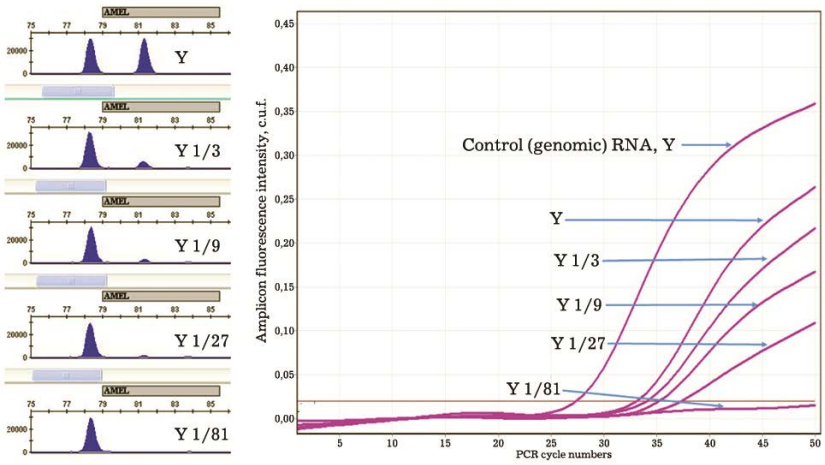

Fig. 6. Examples of fragment analysis data (A) and RT-PCR data (B) for sequential dilutions of male cfDNA into female cfDNA (according to Y-chromosome markers, dilutions of 1/3, 1/9, 1/27, and 1/81)

For one pair, it was also possible to calculate the proportion of male cfDNA using the STR marker D2S441. In Fig. 7A, the results obtained for this marker are shown by the lilac curve. This heterozygous locus had

mismatched alleles in the donors used to prepare the chimeric sample. The length of the target DNA was from 90 to 100 bp.

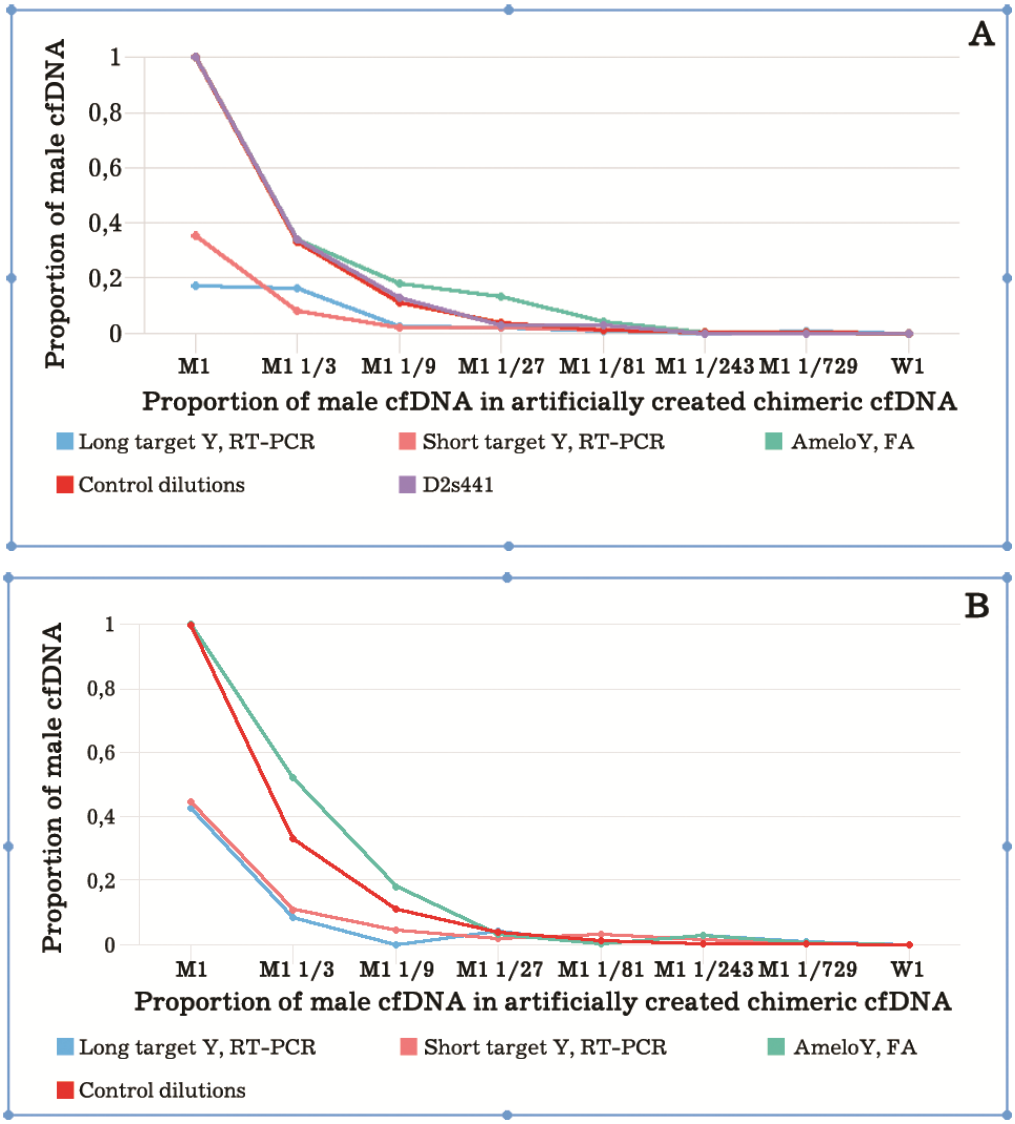

Fig. 7. Fractions of male cfDNA in a dilution sequences from 1 to 1/729. A, donor 1; B, donor 2. RT-PCR, real-time polymerase chain reaction, cfDNA, circulating free DNA; D2s441, allelic STR marker; ameloY, amelogenin Y chromosome

As seen from Fig. 7, we obtained a fairly high agreement in the change of the male cfDNA fraction when testing with different methods. The red curves represent the proportion of the male cfDNA calculated from the dilution results, and the values obtained by FA of short DNA

targets are closest to the red curve in both sets of dilutions from two pairs of healthy donors. However, the analysis of longer DNA targets by PCR detected only about 20–40% of the actual target cfDNA fraction.

Discussion

The obtained results indicate that a shorter target DNA sequence has a higher chance of successful amplification from fragmented DNA. We were able to demonstrate this quantitatively and evaluate the statistical significance of the obtained results using simple models. In Figure 8, we demonstrated possible reasons for the inadequately efficient amplification of target DNA from fragmented DNA. Depending on the primer placement on the fragment, the amplification may occur with varying efficiency or not occur at all. In our work, when analyzing long DNA targets, the amplification efficiency was low and increased up to 10 times with decreasing the target length.

In their review article, A.R. Thierry et al. [19] described a possible mechanism of cfDNA fragmentation. During apoptotic DNA cleavage, a characteristic ladder-like pattern of DNA fragments of 180–200 bp (most) or multiples thereof (oligonucleosomes) is formed. The DNA fragmentation occurs under the impacts of caspase-activated DNase in dying cells and lysosomal DNase II after phagocytosis of dying cells. The nucleosome consists of a histone octamer and double-stranded DNA wrapped around this protein complex, which is stabilized by histone H1. Each nucleosome is linked to another double-stranded DNA (linker). The length of DNA wrapped around the histone octamer is approximately 147 bp, and the length of the linker site is from 20 to 90 bp. These fragments are linked together to ensure the structural integrity of the nucleosome and protect the DNA from enzymatic degradation in the circulatory system. The standard size of cfDNA is thus from 160 to 180 bp. Based on

these data, it is possible to estimate the amplification efficiency of target DNA of different lengths depending on the probable position of the primer on the cfDNA fragment (Fig. 8).

Therefore, to determine the proportion of the target cfDNA fragment, it is important to understand the applicability of the method and the optimal size of the target DNA. Due to the random nature of cfDNA fragmentation, it can be difficult to predict the efficiency of primers for DNA amplification, but shorter targets have a higher chance of successful amplification.

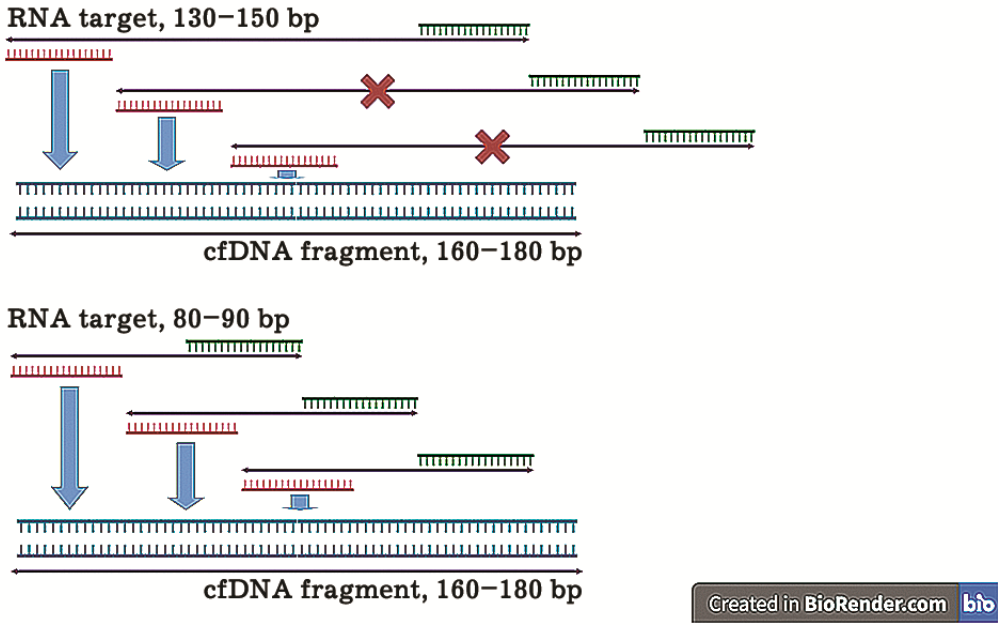

Fig. 8. Size of target DNA and probable primer positions for cfDNA. Blue arrows designate successful primer a**nnealing**; crosses demonstrate amplification failure

Current liver function tests lack sufficient specificity and sensitivity to effectively diagnose most allograft pathologies, including the acute rejection (AR). The “gold” diagnostic standard remains the graft biopsy in case of its dysfunction. In their study, E. Fernández-Galán et al. [20] assessed the possibility of measuring dcfDNA as an AR biomarker (a so-called liquid biopsy). The total concentration and size of cfDNA fragments, as well as the percentage of dcfDNA were monitored in blood

plasma of 20 patients without rejection and 7 patients with T-cell-mediated AR during the first 3 months after liver transplantation. The mean percentage of dcfDNA was 3-fold higher in patients before diagnosing AR and moderately higher in patients with confirmed diagnosis of AR compared to that in patients without subsequent or diagnosed rejection. The proportion of 100–250 bp cfDNA fragments was higher in patients diagnosed with AR compared to that in patients without rejection (68.0% vs. 57.9%, p=0.02). STR-PCR may be an alternative strategy for rapid quantification of dcfDNA, which is easy to implement in clinical laboratories. The results of this pilot study have indicated that dcfDNA levels are elevated very early, even 1–2 weeks before the diagnosis of AR, and therefore may be useful as a prognostic biomarker to improve risk stratification of patients. Similar results were obtained for pediatric patients after liver transplantation [21]. In a review article by H. Andrikovics et al. [22], the authors emphasized that the blood plasma cfDNA was found to have a significant role as a biomarker of allograft damage in any organ transplantation.

At the same time, the clinical significance of plasma cfDNA chimerism testing in allo-HSCT has been less studied. However, in identifying relapses in leukemia patients with complete donor chimerism in the peripheral blood and bone marrow cells, it was found that the plasma cfDNA determination technique was more sensitive than the determination of cellular chimerism [5]. Further study showed that mixed chimerism was detected in cfDNA in a higher percentage of samples than in peripheral blood cells after allo-HSCT. Interestingly, plasma cfDNA-based microchimerism analysis allowed the detection of isolated extramedullary relapses when complete donor chimerism was observed in peripheral blood cells [23]. The cfDNA concentration in the recipient increased not only in relapses, but also in transplant-related

complications, especially in acute graft-versus-host disease (aGVHD). The decrease in the severity of aGVHD symptoms during therapy coincided with a decrease in the cfDNA fraction level in the recipient, while the stabilization or enhancement of aGVHD, on the contrary, was associated with stable or increased levels of recipient cfDNA. This association suggests that recipient cell destruction in target organs in aGVHD may be a source of cfDNA. No correlation was found between the proportion of the recipient cfDNA and the severity of aGVHD (grades I–II versus grades III–IV). Chronic GVHD was not associated with mixed chimerism in cfDNA in patients.

M. Waterhouse et al. [24] reported the clinical utility of monitoring the minimal residual disease and mixed chimerism in cfDNA using droplet digital PCR in 62 patients with myeloid malignancies who had received allo-HSCT. A cutoff value of 18% for cfDNA chimerism distinguished the patients with hematological relapses from those in complete remission after allo-HSCT. Most mutations identified by the targeted NGS panel were detected in cfDNA at the time of relapse and were suitable for monitoring the minimal residual disease. In several cases, mutations were detected in cfDNA earlier than in peripheral blood mononuclear cells. A number of studies have been published aimed at analyzing tumor markers in cfDNA in hematological malignancies and solid tumors [25–30]. It is possible that adjusting the length of the target DNA will change the quantitative characteristics of the tests towards a more accurate assessment of cfDNA markers.

The DNA fragments in blood plasma of pregnant women have been found to be significantly longer than in blood plasma of non-pregnant women. In addition, it has been shown that cfDNA molecules of maternal origin are longer than fetal cfDNA (fcfDNA) molecules of the fetus. K.C.A. Chan et al. found that the proportion of blood plasma cfDNA

fragments larger than 201 bp made 57% in pregnant women and 14% in non-pregnant women. The proportion of fcfDNA fragments larger than 193 bp and larger than 313 bp in maternal plasma was 20% and 0%, respectively [31]. A retrospective cohort study conducted by Y. Hou et al. that included 27,793 women with singleton pregnancies was aimed at identifying sex chromosome aneuploidies and trisomies of chromosomes 18, 13 and 21. It was noted that the cfDNA fetal fraction (FF) in T18 and T13 subgroups was significantly lower than in the group without trisomy, while FF in the T21 group was significantly higher than in the group without trisomy. Pearson correlation analysis revealed a positive correlation between the FF content and the duration of pregnancy in the risk groups [32]. Thus, the importance of the most accurate determination of FF becomes obvious. Detection of fcfDNA in maternal plasma enhanced the development of the NIPT method. Currently, this method is available worldwide and is highly accurate for detecting fetal chromosomal aneuploidy. More than 6 million pregnant women have undergone this procedure to detect fetal aneuploidy. Since the NIPT implementation in practice, several scientific committees around the world have published and updated guidelines and recommendations to support the clinical use of NIPT in pregnant women [33]. Since 2011, NIPT has been used commercially to detect chromosomal aneuploidies. Several clinical studies have shown that FF level is a crucial parameter for NIPT. Y.-S. Lu et al. conducted a large-scale retrospective analysis of the NIPT performance [34]. Pregnant women (n=282,911) who participated in a free-of-charge NIPT (April 2018–December 2021) were screened for common trisomies. The NIPT performance was assessed for its positive predictive value, sensitivity, and specificity. In DNA sequencing by ion semiconductor sequencing, in which DNA was fragmented, then specific DNA adapters were ligated to the ends of the

resulting fragments for the emulsion PCR on magnetic beads and subsequent sequencing; the average length of fragments used to generate a library was about 135–145 bp, and the length of reads was about 200 bp. There were 7 (1%) false negatives.

H.-J. Kwon et al. [35] performed paired-end sequencing to determine the size range of fetal and maternal cfDNA in 62,374 pregnant women. Based on the sequencing results, a criterion was proposed defining fetal cfDNA as less than 150 bp and maternal cfDNA as greater than 180 bp. By implementing the size selection criterion, the accuracy of NIPT was improved, resulting in an increase in the overall positive predictive value for all aneuploidies from 89.57% to 97.10%. This was achieved by enriching both fetal and maternal cfDNA, which increased the proportion of fetal cfDNA; and the false positive rate for all aneuploidies was reduced by more than 70%. E. Shubina and co-authors developed an approach to assessing the FF regardless of the fetus gender by using high-throughput sequencing of a set of frequent single-nucleotide polymorphisms (SNPs). For each of the polymorphisms analyzed in the system, specific primers were selected to amplify the target fragment. The primers were designed so that the length of the PCR product did not exceed 110 bp [36].

Conclusion

The size of the target DNA has a significant impact on the accuracy of cfDNA measurements (donor, fetal, etc.). Reducing the target DNA length to 84 base pairs results in a more efficient detection of target circulating free DNA with an increase in its proportion in total circulating free DNA by up to 10 times ($p<0.05$) in both liver transplant patients and allogeneic hematopoietic stem cell transplant patients, and allows the detection of low-concentration fcfDNA. In cases where a significant

proportion of target free circulating DNA is expected in the sample, the standard genomic DNA analysis methods can be used, but this may require corrections for a reduced sensitivity of the method.

Thus we can make the following conclusions:

1) In liver transplant patients, the mean proportion of male cfDNA was 0.035±0.079, 0.096±0.150 ($p=0.009$, statistically significant) and 0.350±0.388 ($p=0.001$, statistically significant) when using three different PCR analysis testing systems.

2) In the group of patients after allogeneic hematopoietic stem cell transplantation, the mean proportion of male cfDNA was 0.032±0.043, 0.150±0.177 ($p=0.005$, statistically significant), and 0.341±0.308 ($p<0.001$, statistically significant).

3) In the group of pregnant women, when using the standard system of primers for RT-PCR, Y-chromosome markers were not detected; when using the modified system for RT-PCR and the system of primers to short DNA targets for PCR with fragment analysis, the mean proportion of male cfDNA was 0.007±0.013 ($p=0.076$, statistically significant) and 0.077±0.095 ($p=0.014$, statistically significant), respectively.

4) When analyzing artificial chimeras with a known proportion of male cfDNA, the values obtained using fragment analysis of short DNA targets were closest to the actual ones. The analysis of longer DNA targets using real-time PCR revealed only about 20–40% of the target cfDNA fraction.

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