MODIFIED MULTIPLEX REAL-TIME PCR FOR QUANTIFICATION OF DIFFERENTLY SIZED CELL-FREE DNA FRAGMENTS

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There is evidence that size distribution of cell-free DNA (cfDNA) fragments can be diagnostically relevant. The present work describes a multiplex quantitative real-time polymerase chain reaction technique modified and validated by the authors to study the degree of cfDNA fragmentation in blood plasma. Based on the detection of Alu and hLINE-1 repeats, this technique employs fluorescent probes. We selected suitable primers and probes, optimized PCR conditions and estimated the dynamic range and sensitivity threshold of the assay. The modified PCR had a dynamic range of 6 logs, its efficiency being over 90 %. We demonstrated that cfDNA fragmentation index did not differ significantly between healthy women (n = 16) and women with stage III–IV ovarian cancer (n = 14). Therefore, further research on a larger sample is needed using electrophoretic cfDNA fractionation.

Keywords: cell-free DNA, cfDNA, multiplex quantitative PCR, Alu repeats, hLINE-1 repeats, primers, fluorescent probe

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Recieved: 25.08.2017 **Accepted:** 30.08.2017

МОДИФИКАЦИЯ МУЛЬТИПЛЕКСНОЙ ПЦР В «РЕАЛЬНОМ ВРЕМЕНИ» ДЛЯ КОЛИЧЕСТВЕННОЙ ОЦЕНКИ ФРАКЦИЙ ВНЕКЛЕТОЧНОЙ ДНК РАЗНОГО РАЗМЕРА

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Данные о распределении фрагментов внеклеточной ДНК (вкДНК) по фракциям в зависимости от их размера могут иметь диагностическую ценность. В статье описывается разработанный и валидированный авторами метод мультиплексной количественной полимеразной цепной реакции в «реальном времени» для исследования степени фрагментированности вкДНК плазмы крови. Метод основан на определении фрагментов Alu и hLINE-1 с помощью флуоресцентных зондов. Были подобраны последовательности праймеров и зондов, проведена оптимизация ПЦР и оценка динамического диапазона измерений и нижнего порога детекции. Аналитические характеристики метода: динамический диапазон измерений — 6 порядков, эффективность ПЦР — более 90 %. С использованием разработанного подхода было показано, что индекс фрагментированности вкДНК плазмы крови женщин с раком яичников стадий III–IV (n = 14) не отличается достоверно (p = 0,34) от этого показателя у здоровых женщин (n = 16). В дальнейшем следует проводить исследование на более крупной выборке, а также использовать электрофоретическую селекцию фрагментов вкДНК по фракциям.

Ключевые слова: внеклеточная ДНК, вкДНК, мультиплексная количественная ПЦР, Alu-повторы, hLINE-1-повторы, праймеры, флуоресцентный зонд

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Статья получена: 25.08.2017 **Статья принята к печати:** 30.08.2017

An idea was first proposed in 1981 to quantify circulating cell-free DNA (cfDNA) to assess a therapeutic effect of anti-metastatic drugs in xenografts [1]. The method developed by the researchers was based on PCR amplification of human Alu sequences using ^{32}P-labeled 2'-deoxycytidine-5'-triphosphate (dCTP) and had high sensitivity (an equivalent of one human tumor cell could be detected in 1×10^6 murine cells) ensured by a high content of Alu sequences in human genomic DNA. Found in primates, Alu elements are repeated DNA sequences scattered abundantly across the genome, which contains from 500,000 to 1 million of their copies. Being abundant and conserved, human Alu repeats are a perfect target for PCR amplification [2]. In 2002 the Alu-based method of cfDNA quantification was adapted to routine use: it was proposed to use real-time polymerase chain reaction assays with intercalating dyes for signal detection [3, 4]. The advantages

of the modified method prompted the researchers to develop a similar system based on hLINE-1 repeats with 6 kbp-long consensus sequences and a frequency of 200,000 copies per genome [5]. In 2007 an article was published describing an approach to cfDNA quantification by hLINE-1-based real-time PCR [6].

Quantification of circulating cell-free DNA was believed to have the potential for the diagnosis of ovarian cancer. But in spite of a few encouraging findings, generally the method is diagnostically imperfect with regard to a number of important parameters [7]. Perhaps, a ratio of long (>180 bp) to short cfDNA fragments would be a more reliable biomarker of cancer. Longer DNA fragments are assumed to originate from tumor necrosis, changing the ratio of various cfDNA molecules [8].

Using quantitative PCR to amplify variously sized DNA fragments, a few researchers demonstrated that longer fragments make a considerable contribution to the total amount of cfDNA molecules in cancer patients [9, 10]. It was shown that in patients responsive to treatment the proportion of longer fragments decreases during therapy, whereas stably high levels of cfDNA are indicative of poor treatment outcome [9]. However, when amplicon length and tumor-specific somatic mutations were analyzed in another study, it was discovered that tumor cfDNA is shorter [11]. An assumption could be made that a degree of tumor cfDNA fragmentation depends on cancer type and predominant type of cell death.

In this work we describe a multiplex quantitative PCR assay that we designed, validated and used to study the degree of fragmentation of tumor cfDNA in patients with ovarian carcinoma.

METHODS

Our study was conducted in the patients of Blokhin Russian Cancer Research Center. The main group comprised 14 patients with stages III and IV ovarian serous adenocarcinoma, aged 64.3 ± 5.6 years (mean age was 64 years). The control group included 16 seemingly healthy women without cancer aged 60.8 ± 8.2 years (mean age was 60.5 years). The study was approved by the ethics committee of the Center (Protocol No. 3 dated May 16, 2016). Participants' data were anonymised.

DNA was isolated from the blood plasma of the participants. Blood samples of healthy women were collected during routine medical check-ups. Blood was collected into 6 ml EDTA-containing vacutainer tubes. The samples were then centrifuged at 2,000 g for 10 min; plasma was transferred to new tubes and then centrifuged at 2,000 g for 10 min. Centrifuged plasma aliquots were transferred to new tubes and stored at –70 °C. The aliquots were thawed immediately before DNA isolation performed using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Netherlands).

Designing primers and fluorescent probes for Alu and hLINE-1

Oligo primers and a fluorescent probe for Alu amplification were selected based on the alignment of consensus sequences of the most common Alu families, namely Alu Sx, Alu Sp and Alu Y [12] (Fig. 1).

For hLINE-1amplification primer sequences were borrowed from [6]. The design of a fluorescent probe was our own, created in UGENE v1.14 [13] and OligoAnalyzer v1.0.3 (Teemu Kuulasmaa, Finland). To ensure multiplexing, the fluorescent probes for Alu and hLINE-1 contained different dyes. Primer and probe sequences for Alu and hLINE-1 amplification, as well as amplicon lengths, are shown in Table 1.

Detection of cfDNA in blood plasma by real-time PCR

Real-time PCR was performed in the CFX96 Thermal Cycler (Bio-Rad, USA). The volume of the reaction mixture was 25 µL and contained 65 mM Tris-HCl (pH 8.9), 24 mM ammonium sulfate, 3.5 mM magnesium chloride, 0.05 % Tween-20, 0.2 mM dNTP, 0.3 Mm of each primer, 0.1 mM of each fluorescent probe (Table 1) and 1 unit of thermally stable modified Taq polymerase (Interlabservice, Russia). The PCR

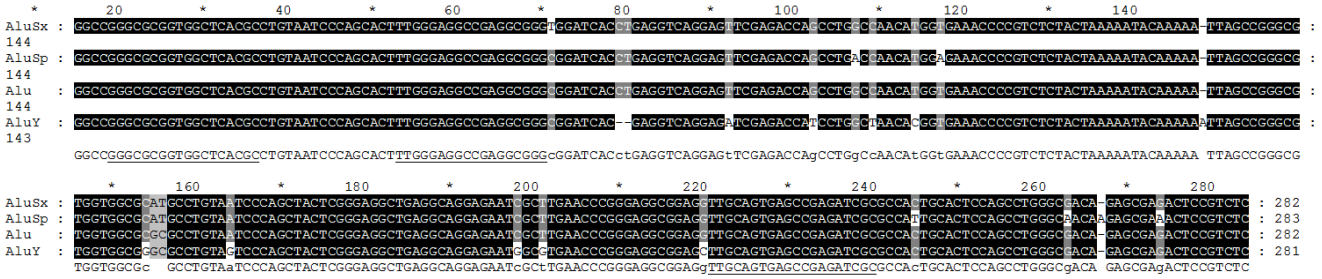

Fig. 1. Alignment of consensus nucleotide sequences of different Alu families. Underlined are positions of the oligonucleotide primers and the fluorescent probe. Positions in which a nucleotide sequence matches a consensus sequence are shown in dark grey (the bottom line); positions in which nucleotide sequences contain substitutions are shown in light grey. Nucleotide deletions are represented by dashes

Table 1. Primers and fluorescent probes for Alu and hLINE-1

Name	Sequence	Amplicon length, bp
Alu-U	5'-GGGCGCGGTGGCTCACGC-3'	235
Alu-R	5'-GCGATCTCGGCTCACTGCAA-3'	
Alu-Pr	5'-FAM-TTGGGAGGCCGAGGCGGG-BHQ-3'	
LN-U	5'-TCACTCAAAGCCGCTCAACTAC-3'	82
LN-R	5'-TCTGCCTTCATTTCGTTATGTACC-3'	
LN-Pr	5'-HEX-TGGAAACTGAACAACCTGCTCCTG-BHQ-3'	

protocol was as follows: 15-min initial denaturation at 96 °C followed by 45 cycles of 10 sec at 96 °C and 30 sec at 58 °C. The optimal annealing temperature was determined empirically.

Dynamic range and sensitivity threshold of multiplex PCR

The initial concentration of double-stranded DNA solution was measured spectrophotometrically at 260 nm wavelength (NanoDrop LITE, Thermo Fisher Scientific, USA). Purity of the sample was evaluated using the A260/280 ratio. The dynamic range and sensitivity threshold were determined based on a series of seven 10-fold dilutions of known concentrations of genomic DNA (80 ng/μL). For each sample measurements were taken in triplicate. The sensitivity of our method was estimated based on the sensitivity threshold, which was defined as the amount of human DNA per 1 μL of the sample.

Statistical analysis

Efficiency of multiplex PCR (E) and its dynamic range of linearity (R^2) were estimated using the Bio-Rad CFX Manager Software v3.0 supplied by the manufacturer of the CFX96 Thermal Cycler. Significance of differences in fraction ratios (R) between the main and control groups was estimated using the non-parametric Mann–Whitney U-test.

RESULTS

Optimization of conditions for multiplex PCR

Conditions for multiplex PCR were optimized by selecting the optimal annealing temperature from the range between 55 °C and 68 °C. The best fluorescence ratio and the maximal efficiency of multiplex PCR were observed at 58 °C.

Dynamic range and sensitivity threshold

Calibration curves were constructed from a series of seven 10-fold dilutions of 80 ng/ μL human genomic DNA (Fig. 2). Measurements were taken in triplicate for each dilution aliquot. The dynamic range of linearity of multiplex PCR was 6 logs (Fig. 3). PCR efficiency for Alu fragments was 94.6 % (R^2 = 0.988), for hLINE-1 — 92.1 % (R^2 = 0.996).

Sensitivity threshold was determined in a series of seven 10-fold dilutions of human genomic DNA at a concentration of 80 ng/μL; measurements were taken in triplicate for each aliqot

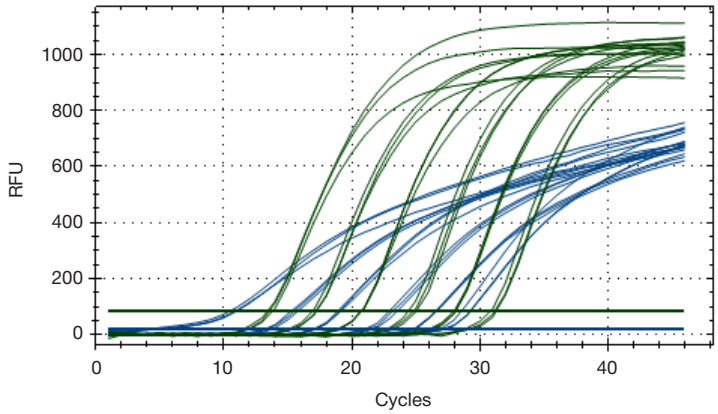

Fig. 2. Multiplex PCR for Alu and hLINE-1 amplification. The X-axis represents PCR cycles, the Y-axis shows fluorescence intensity expressed in RFU. Blue curves represent the FAM probe, green curves represent the HEX probe

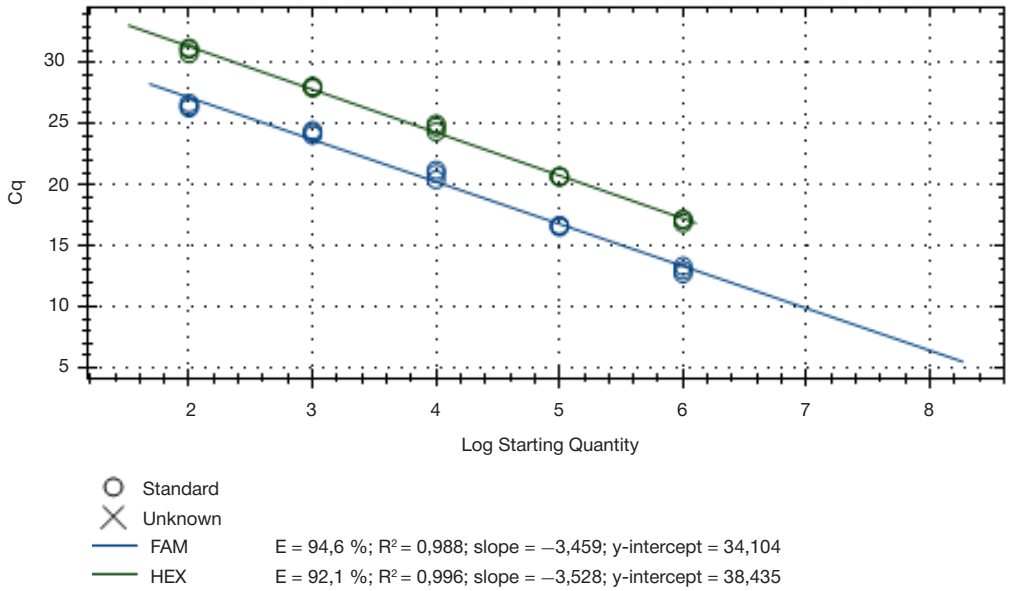

Fig. 3. The dynamic range of the method. The X-axis shows the sample amount expressed in rel. un., the Y-axis depicts quantification cycle Cq. Measurements were taken in triplicate

(ng/μL: A1 = 80, A2 = 8, A3 = 0.8, A4 = 0.08, A5 = 0.008, A6 = 0.0008, A7 = 0.00008, A8 = 0.000008) in the presence of negative control. For both Alu and hLINE-1, the sensitivity threshold of the method was 0.08 pg/μL.

Analysis of cfDNA in the blood plasma of women with ovarian cancer and healthy women

The original method for quantification of Alu and hLINE-1 sequences was applied to estimate the ratio of different cfDNA fractions in the blood plasma of women with ovarian cancer (n = 14) and seemingly healthy women (n = 16). This ratio (R) was calculated by dividing the number of 235 bp Alu fragments expressed in arbitrary units into the number of 82 bp hLINE-1 fragments expressed in arbitrary units. We did not find any significant differences in R values between the groups (Table 2, Fig. 4).

DISCUSSION

This study demonstrates that our original Alu and hLINE-1-based multiplex real-time PCR assay is reliable, accurate and highly sensitive with regard to plasma cfDNA quantification. The method has good analytical characteristics because, firstly, it employs fluorescent probes, in contrast to the existing methods of cfDNA quantification that also make use of Alu and hLINE-1 sequences. This ensures specificity of amplicon detection and becomes an advantage over other methods based on the use of intercalating dies [3, 4, 14]. Secondly, multiplexing becomes possible again due to the use of fluorescent probes. This makes the whole procedure less complicated and less time-consuming. Thirdly, the method has good analytical characteristics: the dynamic range of measurement is 6 logs, PCR efficiency is over 90 %. Fourthly, sensitivity threshold demonstrated by our method is 0.08 pg/μL, which is by 4–5 logs lower than typically achieved concentrations of cfDNA isolated from the blood plasma of healthy individuals. For example, when cfDNA is isolated from 1 ml of plasma using the QIAamp Circulating Nucleic Acid Kit and DNA is eluted into a final volume of 50 μL, sample concentrations range from 0.5 to 1.5 ng/μL [15]. Lastly, the use of variously sized amplicons in multiplex PCR allows us to estimate the degree of fragmentation of tumor cfDNA.

We applied our method on a small sample of female patients with ovarian cancer (n = 14). No significant difference was found between the group of patients and the controls regarding the ratios of different cfDNA fractions (p = 0.34). However, there still may be a difference, because our sample was small and fraction distribution was estimated very roughly by using only 2 reference amplicon sizes of 235 bp and 82 bp. Further research is necessary based on the electrophoretical selection of cfDNA fragments and quantitative PCR to optimize the design of diagnostic systems for the detection of somatic mutations in tumor cfDNA.

CONCLUSIONS

We have designed and validated a multiplex PCR assay for quantification of variously sized cell-free DNA fragments. Our method has good analytical characteristics: a dynamic range of 6 logs and PCR efficiency of over 90 %. Using this method, we have demonstrated that degree of cfDNA fragmentation in women with ovarian cancer and seemingly healthy women does not differ significantly. Further research is necessary with a larger sample size using electrophoretic cfDNA fractionation.

Table 2. Results of the statistical analysis demonstrate the ratio R of long cfDNA fragments to short ones isolated from the blood plasma of women with ovarian cancer and healthy women

Group	Median R	m ± SD	p (Mann–Whitney)
Women with ovarian cancer (n = 14)	2.56	2.9 ± 2.0	0.34
Healthy women (n = 16)	3.08	3.2 ± 1.7	

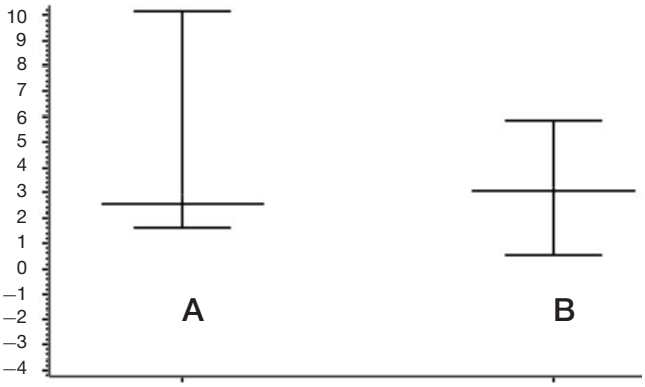

Fig. 4. The ratio R of long cfDNA fragments to short ones isolated from the blood plasma of women with ovarian cancer **(A)** and healthy women **(B)**

References

1. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for Specific Proteases in Cancer Cell Intravasation as Revealed by a Novel Semiquantitative PCR-Based Assay. Cell. 1998 Aug 7; 94 (3): 353–62.
2. Deininger P. Alu elements: know the SINEs. Genome Biol. 2011 Dec 28; 12 (12): 236. DOI: 10.1186/gb-2011-12-12236.
3. Zijlstra A, Mellor R, Panzarella G, Aimes RT, Hooper JD, Marchenko ND et al. A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. Cancer Res. 2002 Dec 1; 62 (23): 7083–92.
4. Schneider T, Osl F, Friess T, Stockinger H, Scheuer WV. Quantification of human Alu sequences by real-time PCR — an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xenotransplants. Clin Exp Metastasis. 2002; 19 (7): 571–82.
5. Boissinot S, Sookdeo A. The Evolution of LINE-1 in Vertebrates. Genome Biol Evol. 2016 Dec 1; 8 (12): 3485–507. DOI: 10.1093/gbe/evw247.
6. Rago C, Huso DL, Diehl F, Karim B, Liu G, Papadopoulos N et al. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. Cancer Res. 2007 Oct 1; 67 (19): 9364–70. DOI: 1.1158/0008-5472.Can-06-0705.
7. Zhou Q, Li W, Leng B, Zheng W, He Z, Zuo M et al. Circulating Cell Free DNA as the Diagnostic Marker for Ovarian Cancer: A Systematic Review and Meta-Analysis. PLoS One. 2016 Jun 2; 11 (6): e0155495. DOI: 10.1371/journal.pone.0155495.
8. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO,

Hesch RD et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001 Feb 15; 61 (4): 1659–65.
9. Umetani N, Giuliano AE, Hiramatsu SH, Amersi F, Nakagawa T, Martino S et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol. 2006 Sep 10; 24 (26): 4270–6. DOI: 10.1200/JCO.2006.05.9493.
10. Ellinger J, Bastian PJ, Haan KI, Heukamp LC, Buettner R, Fimmers R et al. Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. Int J Cancer. 2009 Jan 1; 122 (1): 138–43. DOI: 10.1002/ijc.23057.
11. Mouliere F, Robert B, Arnau Peyrotte E, Del Rio M, Ychou M, Molina F et al. High fragmentation characterizes tumour-derived circulating DNA. PLoS One. 2011; 6 (9): e23418. DOI: 10.1371/journal.pone.0023418.
12. Britten RJ. Evolutionary selection against change in many Alu repeat sequences interspersed through primate genomes. Proc Natl Acad Sci U S A. 1994 Jun 21; 91 (13): 5992–6.
13. Okonechnikov K, Golosova O, Fursov M, the UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012 Apr 15; 28: 1166–7. DOI: 10.1093/bioinformatics/bts091.
14. Lou X, Hou Y, Liang D, Peng L, Chen H, Ma S et al. A novel Alu-based real-time PCR method for the quantitative detection of plasma circulating cell-free DNA: Sensitivity and specificity for the diagnosis of myocardial infarction. Int J Mol Med. 2015 Jan; 35 (1): 72–80. DOI: 10.3892/ijmm.2014.1991.
15. Jung K, Fleischlacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker-a critical appraisal of the literature. Clin Chim Acta. 2010 Nov; 411 (21–22): 1611–24. DOI: 10.1016/j.cca.2010.07.032.

Литература

1. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for Specific Proteases in Cancer Cell Intravasation as Revealed by a Novel Semiquantitative PCR-Based Assay. Cell. 1998 Aug 7; 94 (3): 353–62.
2. Deininger P. Alu elements: know the SINEs. Genome Biol. 2011 Dec 28; 12 (12): 236. DOI: 10.1186/gb-2011-12-12236.
3. Zijlstra A, Mellor R, Panzarella G, Aimes RT, Hooper JD, Marchenko ND et al. A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. Cancer Res. 2002 Dec 1; 62 (23): 7083–92.
4. Schneider T, Osl F, Friess T, Stockinger H, Scheuer WV. Quantification of human Alu sequences by real-time PCR — an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xenotransplants. Clin Exp Metastasis. 2002; 19 (7): 571–82.
5. Boissinot S, Sookdeo A. The Evolution of LINE-1 in Vertebrates. Genome Biol Evol. 2016 Dec 1; 8 (12): 3485–507. DOI: 10.1093/gbe/evw247.
6. Rago C, Huso DL, Diehl F, Karim B, Liu G, Papadopoulos N et al. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. Cancer Res. 2007 Oct 1; 67 (19): 9364–70. DOI: 1.1158/0008-5472.Can-06-0705.
7. Zhou Q, Li W, Leng B, Zheng W, He Z, Zuo M et al. Circulating Cell Free DNA as the Diagnostic Marker for Ovarian Cancer: A Systematic Review and Meta-Analysis. PLoS One. 2016 Jun 2; 11 (6): e0155495. DOI: 10.1371/journal.pone.0155495.
8. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001 Feb 15; 61 (4): 1659–65.
9. Umetani N, Giuliano AE, Hiramatsu SH, Amersi F, Nakagawa T, Martino S et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol. 2006 Sep 10; 24 (26): 4270–6. DOI: 10.1200/JCO.2006.05.9493.
10. Ellinger J, Bastian PJ, Haan KI, Heukamp LC, Buettner R, Fimmers R et al. Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. Int J Cancer. 2009 Jan 1; 122 (1): 138–43. DOI: 10.1002/ijc.23057.
11. Mouliere F, Robert B, Arnau Peyrotte E, Del Rio M, Ychou M, Molina F et al. High fragmentation characterizes tumour-derived circulating DNA. PLoS One. 2011; 6 (9): e23418. DOI: 10.1371/journal.pone.0023418.
12. Britten RJ. Evolutionary selection against change in many Alu repeat sequences interspersed through primate genomes. Proc Natl Acad Sci U S A. 1994 Jun 21; 91 (13): 5992–6.
13. Okonechnikov K, Golosova O, Fursov M, the UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012 Apr 15; 28: 1166–7. DOI: 10.1093/bioinformatics/bts091.
14. Lou X, Hou Y, Liang D, Peng L, Chen H, Ma S et al. A novel Alu-based real-time PCR method for the quantitative detection of plasma circulating cell-free DNA: Sensitivity and specificity for the diagnosis of myocardial infarction. Int J Mol Med. 2015 Jan; 35 (1): 72–80. DOI: 10.3892/ijmm.2014.1991.
15. Jung K, Fleischlacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker-a critical appraisal of the literature. Clin Chim Acta. 2010 Nov; 411 (21–22): 1611–24. DOI: 10.1016/j.cca.2010.07.032.