THE USE OF WILD-TYPE BLOCKING ALLELE-SPECIFIC REAL-TIME POLYMERASE CHAIN REACTION FOR THE ANALYSIS OF SOMATIC MUTATIONS IN RAS GENES OF CIRCULATING FREE DNA ISOLATED FROM THE BLOOD PLASMA OF PATIENTS WITH COLORECTAL CANCER

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Screening for cell-free DNA usually referred to as liquid biopsy holds great promise in cancer diagnosis and treatment. This article presents the results of the analysis of somatic tumor-specific mutations in circulating free DNA (cfDNA) isolated from the blood plasma of patients with stages I–IV colorectal cancer, based on the use of wild-type blocking allele-specific real-time polymerase chain reaction. This technique was specially designed for the analysis of biological specimens containing small amounts of mutant circulating tumor DNA. The study included 46 patients (18 female and 28 male participants) between 48 and 86 years of age (mean age was 67.1 ± 8.8 years). All patients underwent surgical treatment (radical surgery was performed on 85 % of the participants). Besides the molecular genetic analysis of cfDNA isolated from the blood plasma, standard histological staining was performed. Patients' blood samples were collected before the surgery and on day 5 after it to test for *KRAS* and *BRAF* mutations. The applied PCR technique proved to be effective in detecting mutations in the RAS genes in stages II–IV of the disease, its sensitivity threshold being 0.1 %. Analysis of cfDNA before and after surgery may provide additional information on the surgical treatment outcome, development of new metastases, or presence of those previously overlooked. Wild-type blocking allele-specific real-time PCR is awaiting further validation in different clinical situations.

Keywords: liquid biopsies, cell-free DNA, circulating free DNA, circulating tumor DNA, somatic mutation, non-invasive testing, cancer

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АНАЛИЗ СОМАТИЧЕСКИХ МУТАЦИЙ В ГЕНАХ RAS-КАСКАДА СВОБОДНО ЦИРКУЛИРУЩЕЙ ДНК ПЛАЗМЫ КРОВИ ПАЦИЕНТОВ С КОЛОРЕКТАЛЬНЫМ РАКОМ МЕТОДОМ УСИЛЕННОЙ АЛЛЕЛЬ-СПЕЦИФИЧЕСКОЙ ПЦР В «РЕАЛЬНОМ ВРЕМЕНИ»

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Анализ внеклеточной ДНК (жидкостная биопсия) — перспективное направление в современной медицине, особенно в онкологии. В статье представлены результаты исследования соматических онкоспецифических мутаций в свободно циркулирующей ДНК (сцДНК) плазмы крови пациентов с колоректальным раком стадий I–IV методом усиленной аллель-специфической полимеразной цепной реакции в «реальном времени». Названный метод был разработан специально для анализа биологических образцов, содержащих небольшое количество мутантной опухолевой ДНК. В исследование включили 46 человек (18 женщин, 28 мужчин) в возрасте 48–86 лет (средний возраст — 67,1 ± 8,8 года). Все пациенты получили хирургическое лечение (радикальное — в 85 % случаев). Молекулярно-генетическое исследование сцДНК плазмы крови проводили на основе результатов стандартного исследования образцов опухолевой ткани. Кровь отбирали до операции и на 5 день после нее. Анализировали мутации в генах *KRAS* и *BRAF*, которые были выявлены в ткани опухоли. Результаты исследования показали, что изучаемый метод позволяет выявлять мутации в генах RAS-каскада чаще на стадиях II–IV заболевания, а порог его чувствительности составляет 0,1 %. Исследование сцДНК до и после операции предположительно может давать дополнительную информацию о качестве хирургического вмешательства, появлении метастазов или существовании недиагностированных метастазов. Метод усиленной аллель-специфической ПЦР в «реальном времени» должен быть валидирован и оценен в различных клинических ситуациях.

Ключевые слова: жидкостная биопсия, внеклеточная ДНК, свободно циркулирующая ДНК, циркулирующая опухолевая ДНК, соматические мутации, неинвазивная диагностика, онкология

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Precision oncology implies treatment strategies that take into account individual molecular and genetic properties of a patient's tumor. This approach considerably improves treatment outcomes due to the use of therapeutic agents targeting genetic abnormalities in malignant cells. The genetic profile of a tumor has been proved to be unique for each patient, incorporating mutations both in the genes involved in cancer development and randomly occurring across the genome [1, 2].

Tissue samples for molecular genetic analysis are normally collected during surgery or biopsy (prior to treatment), which means that sample collection and subsequent processing can be quite challenging. The diagnostic value of a surgically obtained sample is questionable: typically, tumors are molecularly heterogeneous [3, 4], therefore, a small piece of a tumor cannot accurately represent its molecular genetic profile, let alone the profile of its metastases. Besides, repeated biopsies are labor-intensive and costly.

Tumor tissue specimens are not the only type of biomaterial suitable for molecular genetic analysis. Molecular genetic defects accompanying tumor formation can also be analyzed using patient's blood plasma or serum as the latter contain circulating tumor DNA (ctDNA) regarded as a cancer biomarker [5, 6]. Analysis of cell-free DNA is referred to as liquid biopsy. It helps to circumvent difficulties related to surgical sample collection and can be conveniently used for detection of molecular genetic defects in cancer patients [7]. Blood collection for the analysis is a minimally invasive procedure that can be performed at any time during a therapy course, which makes it possible to monitor any molecular changes in the tumor as they occur [8, 9].

Tumor DNA is found in human blood plasma in low concentrations generally dependent on the disease stage and constitutes less than 1 % of total cell-free DNA [10, 11]. This brings about the necessity of using highly sensitive methods of molecular genetic analysis, such as next generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR). Although their high sensitivity has been confirmed for somatic mutations in cfDNA [12–14], they are not used in clinical routine because of high costs and superfluity of information, as is the case with NGS.

One of the most promising techniques for cfDNA analysis is improved allele-specific PCR developed by Evrogen, Russia, for working with biological material containing small amounts of mutant DNA. The technique combines allele-specific PCR with wild-type allele blocking just like in mutation-specific PCR [15]. Two pairs of primers are selected to amplify a target region that has only one mutation selected for the analysis. Advantageously, this technique yields short PCR products (only 90 b. p. in length), which is important, because ctDNA found in blood plasma is very fragmented. In theory, this technique can be applied to analyze any possible mutations. Currently, it is capable of detecting 7 key mutations in the *KRAS* gene (6 substitutions within codon 12, namely p.G12D, p.G12V, p.G12C, p.G12S, p.G12A, and p.G12R, and one substitution within codon 13, namely p.G13D) and 5 mutations in the *BRAF* gene (p.V600E, p.V600E-2, p.V600K, p.V600K-2, and p.V600D). The sensitivity of this PCR type is at least 10 mutant DNA copies; its selectivity is 0.1–10 % (depending on the amount of initial DNA). The false positive rate is < 0.05 %.

In this work we attempt to use wild-type blocking allele-specific PCR to analyze mutations in the *KRAS* and *BRAF* genes of the RAS family in cfDNA isolated from the blood plasma of patients with colorectal cancer (CRC).

METHODS

The study was conducted in patients with morphologically confirmed carcinomas of the colon or rectum, admitted to the Russian Research Center of Roentgenology and Radiology (Moscow, Russia) over the period from 2010 to 2016.

Patients' tissue samples collected during surgery were analyzed by real-time PCR and then Sanger-sequenced. Based on the results of the analysis, we selected 46 patients with the following activating mutations: exon 2 codons 12 and 13 of *KRAS*; exon 15 codon 600 of *BRAF* [16]. The main group consisted of 46 patients (18 females and 28 males) aged from 48 to 86 years (mean age was 67.1 ± 8.8 years).

Of all participants, 13 (28 %) had stage I cancer, 10 (22 %) had stage II, another 10 had stage III and 13 had stage IV (Table 1). Histologically almost all tumors were adenocarcinomas

Table 1. Distribution of patients with colorectal cancer into groups depending on the levels of cell-free DNA circulating in their blood plasma before surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease stage	Parameter	Patients with detected mutations (n = 24)	Patients without mutations (n = 22)	p-value
I	Number of patients (percentage in the group, %)	3 (12)	10 (45)	–
	Concentrations of cfDNA in blood plasma, ng/μl	3.1 (1.4–3.7)	1.4 (1.2–2.6)	0.09
	Relative amount of mutant ctDNA, %	1.04 (0.14–12.37)	0.02 (0.0–0.03)	0.01*
II	Number of patients (percentage in the group, %)	6 (25)	4 (15)	–
	Concentrations of cfDNA in blood plasma, ng/μl	2.05 (1.6–4.0)	1.4 (0.9–1.8)	0.11
	Relative amount of mutant ctDNA, %	0.47 (0.2–1.9)	0.0 (0.0–0.0)	0.01*
III	Number of patients (percentage in the group, %)	4 (17)	6 (27)	–
	Concentrations of cfDNA in blood plasma, ng/μl	2.4 (1.4–4.9)	1.9 (0.9–1.9)	0.52
	Relative amount of mutant ctDNA, %	2.59 (1.05–10.77)	0.04 (0.0–0.09)	0.01*
IV	Number of patients (percentage in the group, %)	11 (46)	2 (9)	–
	Concentrations of cfDNA in blood plasma, ng/μl	3.8 (1.9–6.9)	1.5 (1.3–1.7)	0.14
	Relative amount of mutant ctDNA, %	5.65 (1.23–20.96)	0.04 (0.0–0.08)	0.03*

Note. Data are presented as median (Q_1–Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

of different grades: 4 patients had poorly differentiated tumors (high grade), 25 — moderately differentiated (intermediate grade), 16 — well differentiated (low grade); 1 patient had a mucin-producing tumor.

All patients underwent surgical treatment. Radical surgery was performed on 85 % of patients (39 individuals), non-radical — on 15 % of patients (7 participants with stage IV cancer). All patients were tested for mutations detected in their tissue samples, namely for p.G12D, p.G13D, p.G12V, p.G12C, p.G12S, and p.G12A of *KRAS* and p.V600E of *BRAF*, which is the most common mutation in CRC. In brief, the protocol was as follows. Before the surgery (n = 46) and 5 days after it (n = 35) patients' blood samples were collected. According to the literature, ctDNA half-life is 15 hours and depends on the location of the tumor, its histological type and disease stage [17, 18]. Thus, blood samples collected on day 5 after the radical surgery would have zero cfDNA in them. Blood was collected into EDTA-containing test tubes (15 ml of specimen per tube). To separate plasma from cell debris, the samples were centrifuged within 1 hour after collection for 15 min at 4 °C in three steps at 1,400, 3,400 and 4,400 rpm, respectively. Plasma aliquots (5 ml) were stored at –80 °C before use.

Circulating DNA was isolated from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The eluate volume was 20 µl for each sample. Concentrations of the isolated DNA were measured by real-time PCR using the XY-Detect kit (Syntol, Russia) according to the manufacturer's protocol.

The *KRAS* and *BRAF* genes were analyzed to check for the presence of the aforementioned mutations by conducting a wild-type blocking allele-specific real-time PCR on the 7500 real-time PCR systems (Applied Biosystems, USA) using reagent kits by Evrogen, Russia. The volume of each cfDNA sample was 10 µl.

Data were statistically processed using Statistica 8 (StatSoft, USA) and Microsoft Excel 2013. Frequency distributions were compared using the Mann–Whitney U.

The study was approved by the Ethics Committee of the Russian Research Center of Radiology and Roentgenology (Protocol No. 3 dated March 17, 2014). All patients gave their informed consent.

RESULTS

The molecular genetic analysis of blood plasma cfDNA performed before surgery revealed the presence of mutations in exon 2 of *KRAS* or exon 15 of *BRAF* in ctDNA of 24 (52 %) patients; the other 22 patients had no such mutations (Table 1). We analyzed how the patients were distributed into subgroups depending on the disease stage and found out that the majority (15 participants, 63 %) of those with mutations in the RAS genes had stages III or IV, while the majority (10 individuals, 45 %) of the patients without mutations in ctDNA had stage I.

Table 1 shows cfDNA concentrations and relative amounts of mutant ctDNA detected in the blood plasma of the participants. Unlike the patients who did not have cancer-associated mutations in the RAS genes detected by wild-type blocking allele-specific PCR, those who did had higher levels of cfDNA regardless of the disease stage, which was particularly noticeable in the subgroups of patients with stage IV cancer. Still, the difference was insignificant due to a high variability of this parameter. In contrast, relative amounts of mutant ctDNA in the blood plasma of patients with cancer-associated mutations were reliably higher than in the participants who did not have these mutations ($p < 0.01–0.03$), their levels of mutant ctDNA being below the sensitivity threshold (0.1 %).

We also analyzed a possible association between the results of our molecular genetic analysis carried out before the surgery and disease progression, metastatic growth and relapse.

Observation time was 27 months. In the group of patients with mutant cfDNA tested before the surgery (n = 24) disease progression was registered in 19 (79 %) individuals; 15 of them died later (Table 2). In the second group (n = 22) 17 patients stayed alive throughout the observation period, but 5 had disease progression and subsequently died. Table 2 provides information about cfDNA levels and relative amounts of ctDNA in the blood plasma of the patients. Both parameters were significantly higher in the patients with detected ctDNA mutations and progressing cancer than in the patients with undetected mutations and progressing cancer. In the group of patients with undetected mutations levels of cfDNA and mutant ctDNA were low, which might explain why the studied PCR technique had failed to detect the mutations. At the same time, our PCR technique effectively detected mutations in the RAS genes in 5 patients without disease progression, in spite of low levels of cfDNA and ctDNA in their blood plasma.

Of 46 participants, cfDNA samples of 35 patients were analyzed both before the surgery and on day 5 after it (Table 3). Of those with detected mutations, 13 individuals (76 %) had disease progression, and 9 (53 %) had stage IV cancer (5 of them underwent nonradical surgery). Apparently, the presence or absence of cancer-associated mutations in cfDNA can indicate how radical the surgery was: in patients with detected mutations high levels of mutant ctDNA may imply that incision of the primary tumor or its metastases was incomplete or that some metastatic lesions were overlooked.

In the group of patients with undetected mutations in the RAS genes, 14 people (78 %) were alive throughout the entire observation period; in another 4 individuals the disease

Table 2. Progression of colorectal cancer in patients characterized by cfDNA levels in their blood plasma measured prior to surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease progression	Parameter	Patients with detected mutations (n=24)	Patients without mutations (n = 22)	p-value
Yes	Number of patients (percentage in the group, %)	19 (79)	5 (23)	–
	Concentrations of cfDNA in blood plasma, ng/µl	3.7 (1.9–6.7)	1.3 (0.9–1.7)	0.01*
	Relative amount of mutant ctDNA, %	1.9 (0.85–14.59)	0.0 (0.0–0.08)	0.0007*
No	Number of patients (percentage in the group, %)	5 (21)	17 (77)	–
	Concentrations of cfDNA in blood plasma, ng/µl	1.7 (1.6–2.2)	1.7 (1.2–1.9)	0.64
	Relative amount of mutant ctDNA, %	0.36 (0.21–3.30)	0.01 (0.00–0.04)	0.0009*

Note. Data are presented as median (Q_1—Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

Table 3. Progression of colorectal cancer in patients characterized by cfDNA levels in their blood plasma measured after surgery and detection of cancer-associated mutations of the RAS genes by allele-specific real-time PCR

Disease progression	Parameter	Patients with detected mutations (n = 17)	Patients without mutations (n = 18)	p-value
Yes	Number of patients (percentage in the group, %)	13 (76)	4 (22)	–
	Concentrations of cfDNA in blood plasma, ng/μl	5.4 (3.5–12.7)	8.65 (5.35–11.50)	0.69
	Relative amount of mutant ctDNA, %	2.15 (0.19–8.43)	0.0 (0.0–0.01)	0.003*
No	Number of patients (percentage in the group, %)	4 (24)	14 (78)	–
	Concentrations of cfDNA in blood plasma, ng/μl	4.75 (2.35–6.95)	5.8 (4.9–8.0)	0.37
	Relative amount of mutant ctDNA, %	0.23 (0.16–0.42)	0.02 (0.0–0.07)	0.003*

Note. Data are presented as median (Q_1–Q_3). Significance of difference was tested by comparing groups of patients with and without mutations in the RAS genes.

* represents significant difference.

progressed. In this group 7 patients had stage I, 5 — stage II, 5 — stage III and 1 — stage IV cancer.

DISCUSSION

It is known that cancer progression is accompanied by increasing levels of cfDNA in blood regardless of tumor location [19]. There is evidence indicating an association between cfDNA levels circulating in blood and clinical manifestations of the disease [20]. Increased cfDNA concentrations are observed in the early stages of tumor formation and can surge in metastasis [21], still varying considerably in different patients [10]. This is unsurprising because cfDNA appears in blood not only when tumor cells or surrounding tissue die, but also as a result of natural degradation of blood cells. Fragments of nucleic acids, including those amplified, secreted by tumor cells also contribute to the total cfDNA circulating in blood. It is known that amplified genome regions are not rare in cancer. Thus, cfDNA concentrations will vary in cancer patients rendering impossible the use of cfDNA as a biomarker.

Concentrations of circulating tumor DNA can be inferred by analyzing cancer-associated mutations. But it should be kept in mind that heterogeneity of the tumor may result in lower mutant ctDNA levels differing considerably from total ctDNA levels [22]. Therefore, ctDNA is not always possible to detect in blood plasma, especially in the early stages of the disease. This may lead to false negative results and reduce sensitivity of the method used for cfDNA analysis. Our findings confirm this hypothesis.

Rachiglio et al. [13] studied ctDNA of 44 patients with non-small-cell lung cancer and 35 patients with colorectal cancer. In their work, the researchers demonstrated the potential of NGS and droplet digital PCR. Using NGS, they were able to detect *EGFR* mutations in the cfDNA of 77.3 % of patients with non-small-cell lung cancer. The mutations were identical to those found in patients' tumor tissue samples. The same mutations were detected in the cfDNA of 2 patients with wild type tumor *EGFR*. Digital PCR confirmed the presence of these mutations both in the primary tumor and blood plasma of these 2 patients. In the same study, mutations in the *KRAS* gene detected by standard PCR techniques before the surgery were confirmed by NGS for cfDNA circulating in the blood plasma of 100 % patients (6/6). At the same time, post-operative NGS detected mutations in only 46.2 % (6/13) of patients. Rachiglio et al. believe that the method they studied is highly sensitive with regard to ctDNA mutations in blood plasma, but its sensitivity depends on the presence of malignant lesions and heterogeneity of driver mutations.

In another study, blood plasma and tumor tissue samples of 58 patients with non-small-cell lung cancer were analyzed by targeted sequencing in order to check for somatic driver mutations [12]. Common driver mutations in the *EGFR*, *KRAS*, *PIK3CA* and *TP53* genes and some rarer mutations found in other genes were detected in blood plasma ctDNA and tumor tissue DNA; the concordance of the method was 50.4 %, sensitivity and specificity were 53.8 % and 47.3 %, respectively. The researchers noted that cfDNA levels correlate with some clinical characteristics of the patients, including disease stage and tumor subtype.

In the work by Tu et al. [14] droplet digital PCR demonstrated a 73 % concordance regarding detected mutations between plasma and tissue samples of 19 patients with colorectal cancer.

To sum up, our findings and the data available in the literature indicate that liquid biopsy based on the analysis of ctDNA levels in blood plasma can be used as an additional diagnostic tool in cancer treatment, mainly in the late stages of the disease or when biopsy cannot be performed. Today, the clinical significance of cfDNA analysis is determined by its role as a prognostic tool in the monitoring of patients. Using wild-type blocking allele-specific PCR performed before the surgery and on day 5 after it, we have demonstrated cancer progression in patients with mutations in ctDNA. By analyzing cfDNA found in blood plasma before and after treatment, we can infer how aggressive the tumor is or whether metastatic growth is present, evaluate the effect of the treatment and make corrections to the treatment plan if the patient is unresponsive.

CONCLUSIONS

There are still difficulties that prevent the use of liquid biopsy in clinical routine. Specifically, there is a need for cheap but highly sensitive methods of analysis of cfDNA circulating in the blood plasma of cancer patients. Preliminary results of our study conducted in patients with stages I to IV colorectal cancer show that wild-type blocking allele-specific real-time PCR is more effective in detecting cancer-associated mutations in the late stages of the disease. Perhaps, this technique will once find its place among the molecular diagnostic tools used in cancer research. It is yet to be validated and assessed in different clinical situations.

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