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HEAD ARTICLE

KLİNİK TƏDQİQATLAR

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SÜD VƏZİSİ XƏRÇƏNGİ OLAN XƏSTƏLƏRDƏ MUTANT PIK3KA – SİRKULYASİYA EDƏN ŞİŞ DNT-si

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Xülasə. *Yerli yayılmış süd vəzisi xərçəngi (SVX) olan xəstədə PIK3KA (H1047R) geninin mutasiyalarının əmələgəlmə tezliyi zəncirşəkilli polimeraza reaksiyası (ZPR) vasitəsilə tədqiq edilmişdir. Bu mutasiyalar şiş toxumalarının arxiv nümunələrində və qan plazmasında (sirkulyasiya edən şiş DNT molekullarının tədqiqi vasitəsilə) öyrənilmişdir.*

Tədqiqata cəlb edilən 29 xəstədən 5 nəfərdə (17%) PIK3KA geninin mutasiyası aşkar edilmişdir. Mutant DNT-yə həm biopsiya materiallarında, həm də qan plazmasında rast gəlinmişdir. Xəstələrin qan plazması nümunələrinin tədqiqi müalicənin müxtəlif mərhələlərində sirkulyasiya edən şiş, DNT-sini aşkar etməyə imkan vermişdir. Bu dəyişikliklər şiş prosesinin dinamikası haqqında məlumat verə bilir.

Tədqiqat göstərmişdir ki, süd vəzisi xərçənginin PIK3KA mutant forması üçün estrogen-mübət və Her2/neu-protein-mənfi fenotipli şişlər daha artıq dərəcədə səciyyəvidir.

Açar sözlər: *PIK3KA, süd vəzisi xərçəngi, sirkulyasiya edən şiş DNT-si*
Ключевые слова: *PIK3CA, рак молочной железы, циркулирующая опухолевая ДНК*
Key words: *PIK3CA, breast cancer, circulating tumor DNA*

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PIK3CA-MUTANT CIRCULATING TUMOR DNA IN PATIENTS WITH BREAST CANCER

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During the research using digital PCR method, tissue and plasma samples from patients with locally advanced breast cancer were examined for the presence of somatic mutation of PIK3CA in biopsy material and circulating tumor DNA. The presence of PIK3CA gene mutation was detected in 17.24% (5/29) of patients in both biopsy material and plasma. A research of serial samples of patients' plasma revealed changes in the amount of circulating tumor DNA throughout all stages of treatment, which reflected the dynamics of the tumor process. For PIK3CA-mutant breast cancer, ER-positive and Her2/neu-negative tumor phenotypes are characteristic.

Malignant tumors occupy a significant place in the structure of general morbidity and mortality, both in Ukraine and around the world [1-3]. Various precancerous and cancerous processes of the reproductive system are an important part of research to create preventive measures and improve treatment regimens [2-5]. A lot of attention is paid to the problem of oncology, but still there are unresolved issues to reduce the incidence of malignant tumors of various localization and mortality from them. The complexity of the situation lies in the delay in identifying the early stages of the primary

tumor process, in the absence of effective ways of prevention and targeted therapy [6-7]. A separate question is about the instability of the tumor genome, both in the primary focus and in distant metastases. At some locations of the tumors, it is impossible to obtain a tissue biopsy to verify the clinical diagnosis and establish molecular genetic characteristics of the tissue for targeted and systemic antitumor therapy [6-8].

The widespread introduction of a minimally invasive and highly sensitive method of "liquid biopsy" can help overcome diagnostic difficulties by obtaining information about the composition and set of mutations in neoplastic tissue by studying extracellular circulating tumor DNA (ctDNA) in various biological fluids [9-10]. The clinical application of detection of ctDNA by the liquid biopsy method has been widely studied in recent years [11-12]. CtDNA, which carries specific genetic changes for tumor, is secreted by neoplastic cells into the bloodstream and represents only a small share of cell-free DNA [11,13-14]. The ctDNA fraction among cell-free DNA varies widely from 0.01% to 90% depending on the tumor burden. Until recently, the limitations of its detection, including a short half-life, are leveled with the development of modern molecular research methods. Based on this, the study of ctDNA has become a promising tool for the diagnosis of malignant diseases. Thus, digital polymerase chain reaction (dPCR) is widely used as one of the most accurate methods for detecting ctDNA in the blood due to its high sensitivity [15].

The PI3K/Akt signal cascade is one of the most important regulators of many processes in normal cells (growth, proliferation, mobility, survival, and apoptosis). Violation of their regulation is observed in a wide range of tumors by enhancing or losing the functions of some components of its signaling pathway, including PTEN, AKT, and PIK3CA [16-19]. Somatic mutations of the PIK3CA gene, which encodes the catalytic subunit of phosphatidylinositol-3-kinase p110-alpha, have been detected in many human malignancies and have important clinical significance in their course [20-21]. In particular, the c.3140A>G (H1047R) mutation in the kinase domain of exon 20, the so-called "hotspot" mutation, has a proven oncogenic effect in different locations of tumor processes [20, 22-24]. Unfortunately, the effect of this DNA modification on the immunophenotype of breast cancer cells remains unclear.

The aim of research - is to determine the frequency of occurrence of a mutation in the PIK3CA (H1047R) gene in patients with locally advanced breast cancer (BC) using dPCR; a study of the comparability of the detection of this mutation in archival samples of tumor tissue (FFPE blocks) and ctDNA of blood plasma; a study of the diagnostic and prognostic value of PIK3CA-mutant ctDNA in patients as a marker for monitoring the effectiveness of treatment.

Material and methods. At this stage, a research included 29 patients with histologically verified breast cancer, who received treatment at the chemotherapy and surgical department of the Sumy Regional Clinical Oncology Center in 2019-2020. All patients underwent a full examination, which included standard clinical laboratory and X-ray studies, computed tomography of the organs of the chest, abdominal cavity and brain. Treatment was prescribed in accordance with standard clinical protocols. All patients signed informed voluntary consent for additional biopsy examination and blood sampling for further PCR testing.

Blood sampling was carried out according to the following schedule: every 2 cycles of neoadjuvant chemotherapy, before and after surgery, before and during adjuvant chemotherapy (every 2 cycles), after completion of treatment during the observation period (every 3 months). After surgical treatment, blood sampling was performed after 24 hours, in 5 and 10 days, which is associated with the half-life of circulating tumor DNA and is considered as the optimal time.

To assess the morphological features of tumor processes in the mammary gland, we performed a histological and immunohistochemical study of the material. The removed tissue was placed in a 10% solution of neutral formalin for 24 hours, dehydrated in xylenes and alcohols (96–70%) in increasing concentrations and contained in paraffin. Thin sections (4 μm) were made from paraffin blocks using a Shandon Finnesse 325 rotary microtome (Thermo Fisher Scientific, USA), placed on glass (Thermo Fisher Scientific, USA) and dried for 12 hours in a thermostat. Then, the procedure was carried out dewaxing and rehydration in xylenes and in descending concentrations of ethanol. Some preparations were stained with hematoxylin and eosin (hematoxylin

staining, inter-stage washing in water, differentiation of structures in 1% hydrochloric acid, incubation with eosin, removal of water in alcohols in increasing concentrations (70–96%) and xylene, exposure in carbene-xylene with the final coating of stained tissue sections with coverslips using a Canada balsam (Thermo Fisher Scientific, USA).

Another part of the drugs was investigated using immunohistochemical methods. The material was dewaxed and dehydrated in xylene and alcohols at lower concentrations (96–70%); thermal unmasking of antigens in a WB-4 water bath (970C) in a pH 6.0 citrate buffer (Thermo Fisher Scientific, USA); visualization of the antigen-antibody reaction by using the detection system "UltraVision Quanto Detection System HRP DAB Chromogen" (Thermo Fisher Scientific, USA), which included blocking endogenous peroxidase with hydrogen peroxide (3% H_2O_2), blocking the non-specific background signal "Ultra V block", strengthening reaction of Primary Antibody Amplifier Quanto, final visualization with diaminobenzidine (DAB); additional staining of the nuclei with Mayer hematoxylin (Thermo Fisher Scientific, USA) and coating the sections with coverslips using a Canada balsam (Thermo Scientific, USA).

Antigen-antibody reaction was studied using the following immunohistochemical markers (Thermo scientific, USA): monoclonal rabbit antibodies clone SP1 and YR85, respectively, to determine the expression of estrogen receptors (ER) and progesterone (PR). Evaluation of the expression of ER and PR was carried out according to the recommendations of D. C. Allred, taking into account the percentage of stained nuclei and the intensity of their color; monoclonal rabbit antibodies (clone SP3) were used to determine HER2/neu oncoproteins, which were evaluated taking into account the completeness and intensity of the color of the tumor cell membrane.

To determine the somatic mutation of the PIK3CA gene (H1047R) at the screening stage, sections of tumor tissue fixed in formalin and embedded in paraffin were used. Then, for patients with a detected mutation in a tissue sample, the mutation of the PIK3CA gene in ctDNA from the blood plasma of patients was determined.

To isolate DNA, tumor tissue from paraffin blocks was used, purified from excess paraffin, weighing up to 25 mg for each sample. DNA from FFPE blocks was isolated using the Quick-DNA FFPE Kit (Zymo Research, USA) according to the manufacturer's protocol. To set up the reaction, eluted DNA was used immediately or stored at $t \leq -20^{o}C$ for future use.

DNA was isolated from blood plasma using the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (Applied Biosystems, USA) according to the protocol provided by the manufacturer.

The concentration of extracted DNA was evaluated using a Qubit 4 Fluorometer and Qubit 1X dsDNA HS Assay Kit, Qubit dsDNA BR Assay Kit (Invitrogen, USA).

Digital PCR was performed using the QuantStudio 3D Digital PCR System (Applied Biosystems, USA), which includes everything necessary for the digital PCR procedure - from loading the test sample into the chip to analyzing the results, namely: a thermal cycler ProFlex 2x Flat PCR System, QuantStudio 3D Digital PCR Instrument (chip reader), QuantStudio 3D Digital PCR Chip Loader. The QuantStudio 3D Digital PCR 20K chip consists of individual 20 000 wells, where each well is a chamber for an individual PCR reaction. Samples of genomic DNA (gDNA) are diluted to the limit in such a way that most individual PCR reactions contain either zero or one DNA molecule [25-26]. The somatic mutation of the PIK3CA gene, represented by the "hotspot" variant in exon 20 of the kinase domain (p.H1047R), was found by using TaqMan dPCR Liquid Biopsy Assay (Applied Biosystems, USA). After mixing a diluted gDNA sample with pre-mixed primers, probes (labeled with FAM and VIC dyes, Applied Biosystems) and PCR master mix, the reaction mixture is loaded onto a chip, which, in turn, is placed in a PCR machine to start the reaction [25]. The thermal cycling protocol is as follows: 10 minutes at 96 ° C, 39 cycles at 60 ° C for 2 minutes, 98 ° C for 30 seconds and 60 ° C for 1 minute. Digital PCR is an endpoint analysis based on the use of the 5'-exonuclease activity of polymerase and TaqMan fluorogenic probes aimed at mutation. The absolute quantification of the target is calculated according to the Poisson statistics based on the number of negative cells that do not contain DNA [25-26]. After setting up the reaction, the chip is placed in QuantStudio 3D Instrument to read fluorogenic signals. Data is analyzed by using QuantStudio 3D AnalysisSuite software. The results are evaluated in copies/µl of nucleic acid sequences labeled with VIC or FAM dyes [25].

This research has been performed with the financial support of grants of the Ministry of Education and Science of Ukraine № 62.14-01.18/20.3П «The effectiveness of "liquid biopsy" and tissue biopsy in the diagnosis and treatment of malignant tumors». The manuscript results are part of the research work of the Department of Oncology and Radiology and Department of Pathology of Sumy State University.

Results. In 17.24% of patients (5/29 cases), a PIK3CA gene mutation was detected in the tumor tissue, which was also found in blood plasma ctDNA. To study the sensitivity and specificity of the dPCR method, plasma samples from 5 patients with breast cancer without the PIK3CA mutation were also tested for PIK3CA-mutated ctDNA. None of these samples showed PIK3CA-mutant ctDNA.

The results of quantitative determination of the PIK3CA mutation using the QuantStudio 3D Digital PCR 20K Chip, were analyzed by using the QuantStudio 3D Digital PCR Analysis Suite Software and presented in Fig. 1.

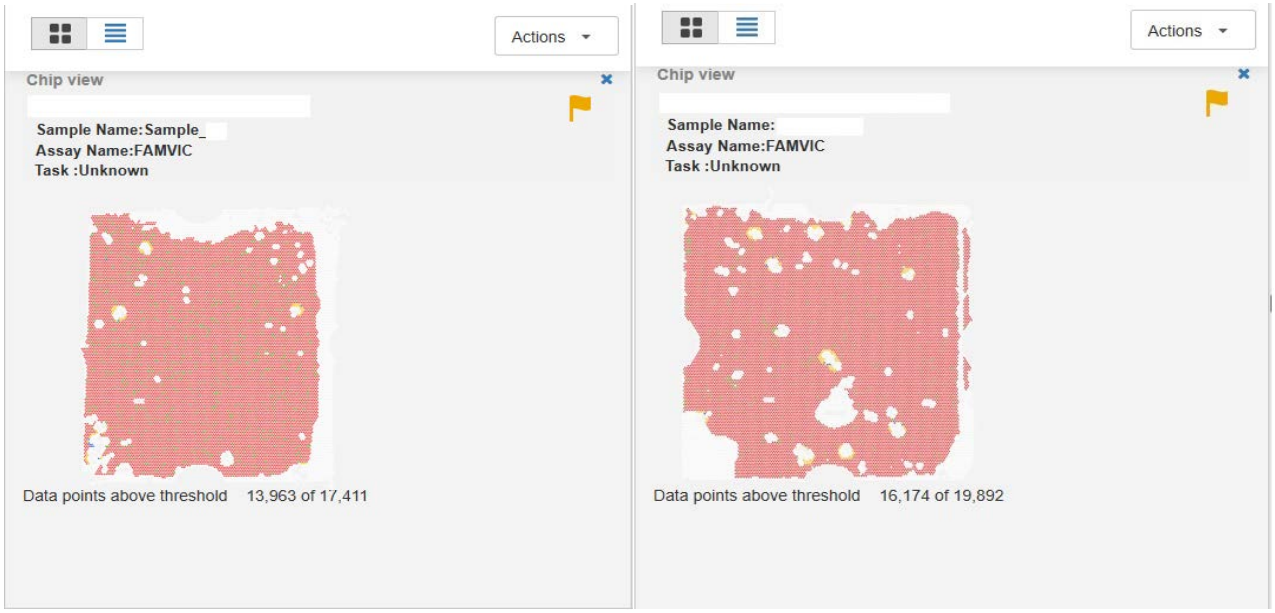
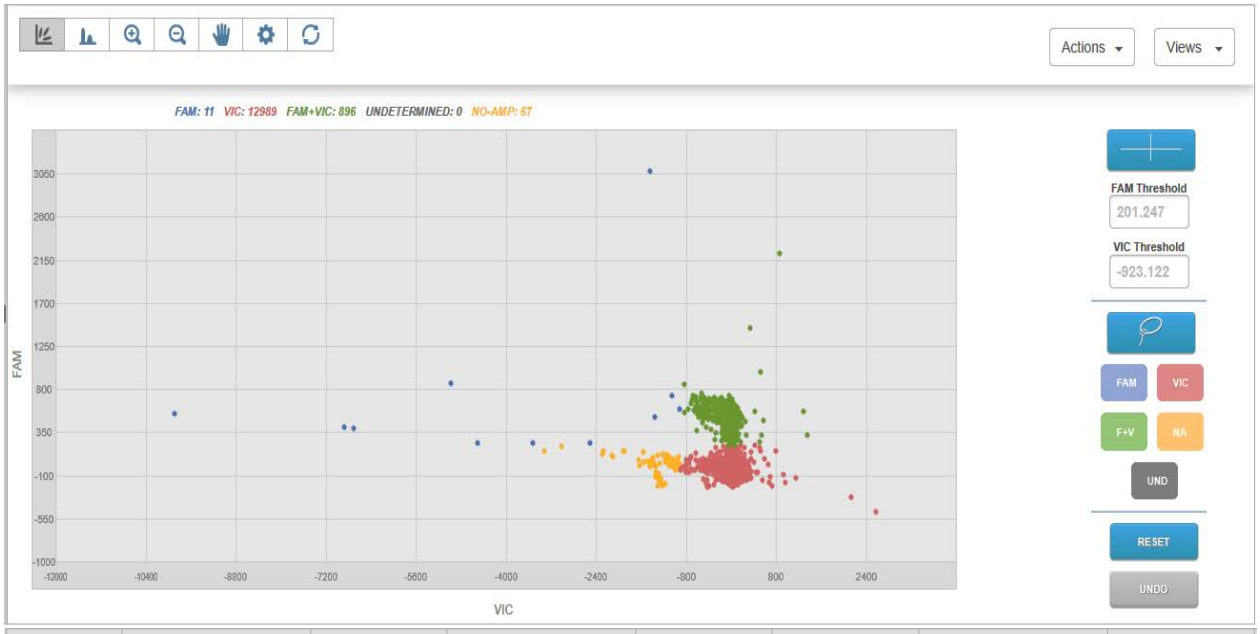


Fig. 1. *The QuantStudio 3D Digital PCR AnalysisSuite Software images of PIK3CA mutated chips with the values of the threshold level in a biopsy sample and patient's blood plasma*

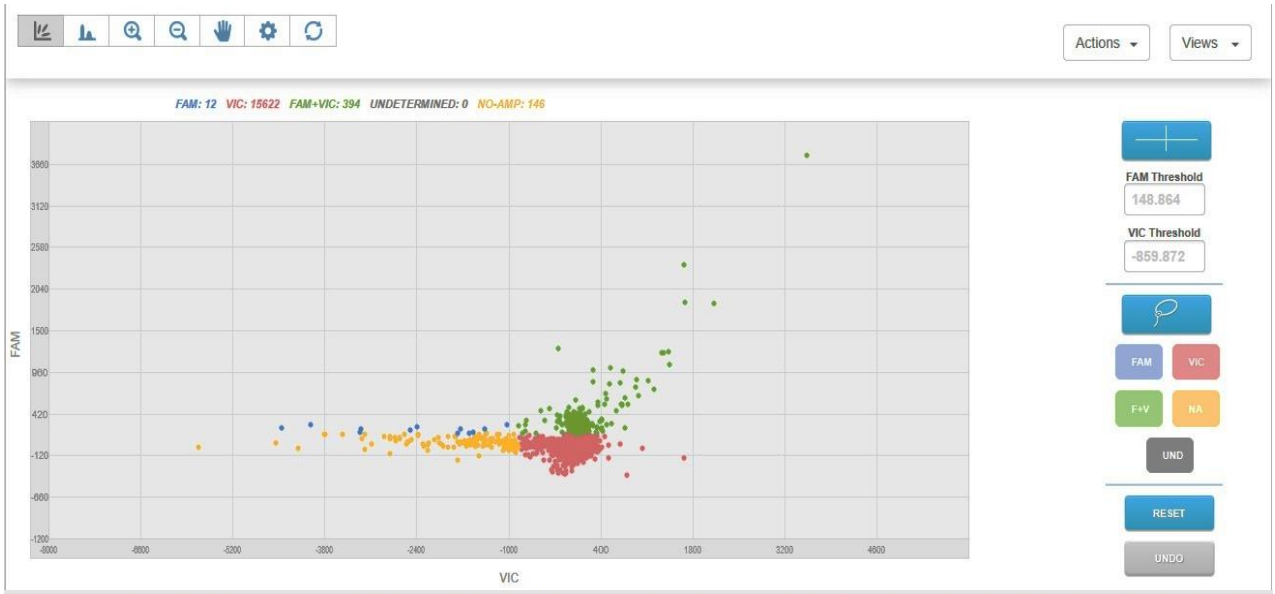


Target/Total	CI Target/Total	Copies/µL (VIC)	CI Copies/µL (VIC)	Precision (VIC)	Copies/µL (FAM)	CI Copies/µL (FAM)	Precision (FAM)
1.263%	1.032% – 1.499%	6957.5	6663.1 – 7265	4.419%	88.972	83.365 – 94.958	6.727%

Fig. 2. *Scatter of data and quantitative indicators in determining the PIK3CA mutation of a biopsy sample of a breast tumor. The analysis scale performed according to the following indicators: Target/Total; CI Target/Total; Copies/uL (VIC); CI Copies/uL (VIC); Precision (VIC); Copies/uL (FAM); CI Copies/uL (FAM); Precision (FAM).*

The obtained reaction results are then displayed in the form of a data scatter diagram showing fluorescence signals: blue FAM signals indicate mutant alleles, red VIC signals indicate wild-type alleles, green signals indicate a combination of mutant and wild type in one well, yellow signals - wells without amplification [25] (Fig. 2 and 3).

These methods allowed us to obtain a quantitative assessment of the PIK3CA mutation in DNA isolated from biopsy tissue (Fig. 2) and the blood plasma of patients (Fig. 3). Data are



Target/Total	CI Target/Total	Copies/µL (VIC)	CI Copies/µL (VIC)	Precision (VIC)	Copies/µL (FAM)	CI Copies/µL (FAM)	Precision (FAM)
0.309%	0.168% – 0.454%	10778	9896.3 – 11738	8.909%	33.452	30.346 – 36.878	10.239%

Fig. 3. *The scatter of data and quantitative indicators in determining the PIK3CA mutation of a plasma sample of a patient with a breast tumor. The analysis scale performed according to the following indicators: Target/Total; CI Target/Total; Copies/uL (VIC); CI Copies/uL (VIC); Precision (VIC); Copies/uL (FAM); CI Copies/uL (FAM); Precision (FAM).*

presented as the number of copies per microliter for the mutant and wild type alleles (average 396.4 and 7457.98 copies / microliter in biopsy samples, as well as 92.9 and 8054.6 copies / microliter in blood plasma samples at the screening stage).

Among patients with detected PIK3CA-mutant ctDNA (5 cases from 29 patients), immunohistochemical studies revealed positive expression of ER in 4 (80%) cases (negative in 1 case – 20%), and positive expression of PR in 3 (60%) cases (negative in 2 cases – 40%). A positive reaction was considered in the presence of more than 1% of tumor cells with nuclear staining DAB (brown color). Her2/neu-positive expression (intense membrane staining in more than 10% of tumor cells) was observed in only 1 (20%) sample (negative in 4 cases – 80%). On the other hand, tumor tissues from patients without mutation (24 cases from 29 patients), were ER-positive in 18 (75%) cases (negative in 6 cases – 25%), PR-positive in 15 (62.5%) cases (negative in 9 cases – 37.5%), and Her2/neu positive in 5 (20.83%) cases (negative in 19 cases – 79.17%). Based on this, it can be argued that for PIK3CA-mutant breast cancer, ER-positive and HER2-negative tumors are more characteristic. Immunohistochemical characteristics of tumors expression are presented in Fig. 4.

A study of serial plasma samples of patients with PIK3CA-mutant breast cancer showed that changes in the amount of ctDNA occur throughout all cycles of neoadjuvant chemotherapy, which reflect the dynamics of disease regression. Assessment of the response to treatment, which based on the dynamics of blood plasma of ctDNA, is comparable to CT data performed at the same time intervals. In the diagram shown in Figure 5, a decrease in the amount of ctDNA can be observed during all cycles of neoadjuvant chemotherapy and after surgery. The lowest concentration is observed on the 1st and 5th day after surgery. In one patient in a plasma sample taken on the 5th day after surgery, PIK3CA-mutant ctDNA was completely absent.

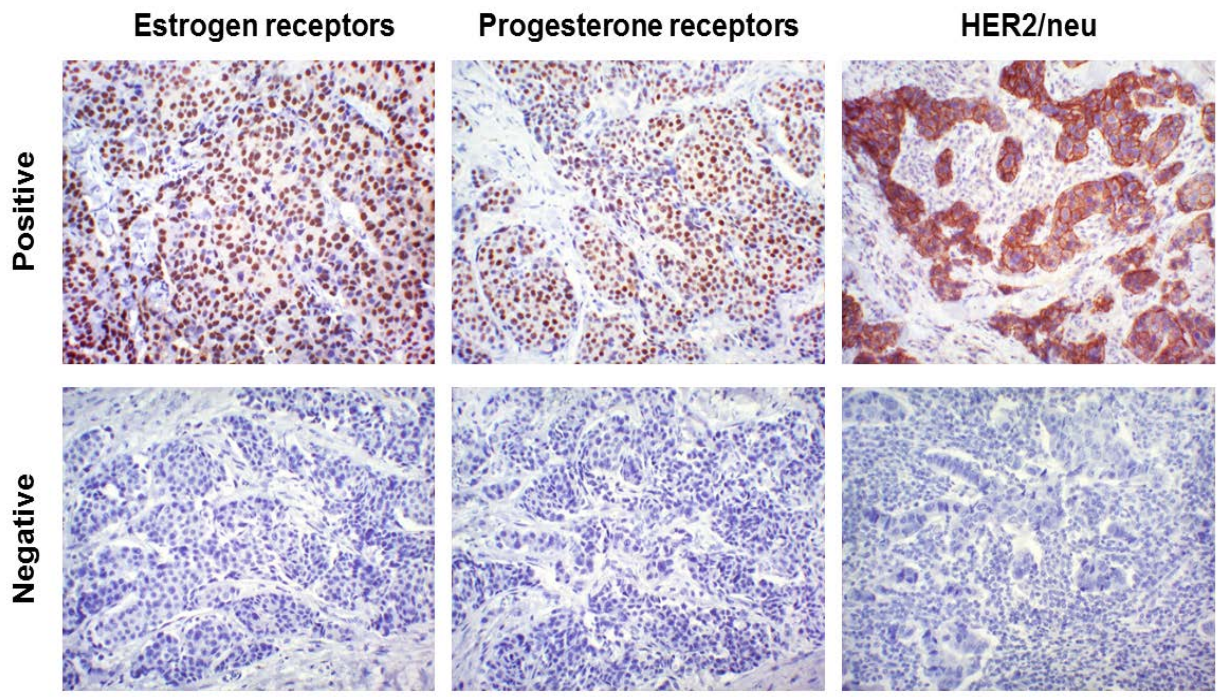


Fig. 4. *Breast tumor. Immunohistochemical staining of Estrogen receptors, Progesterone receptors and Her2/neu. Magnification - x200.*

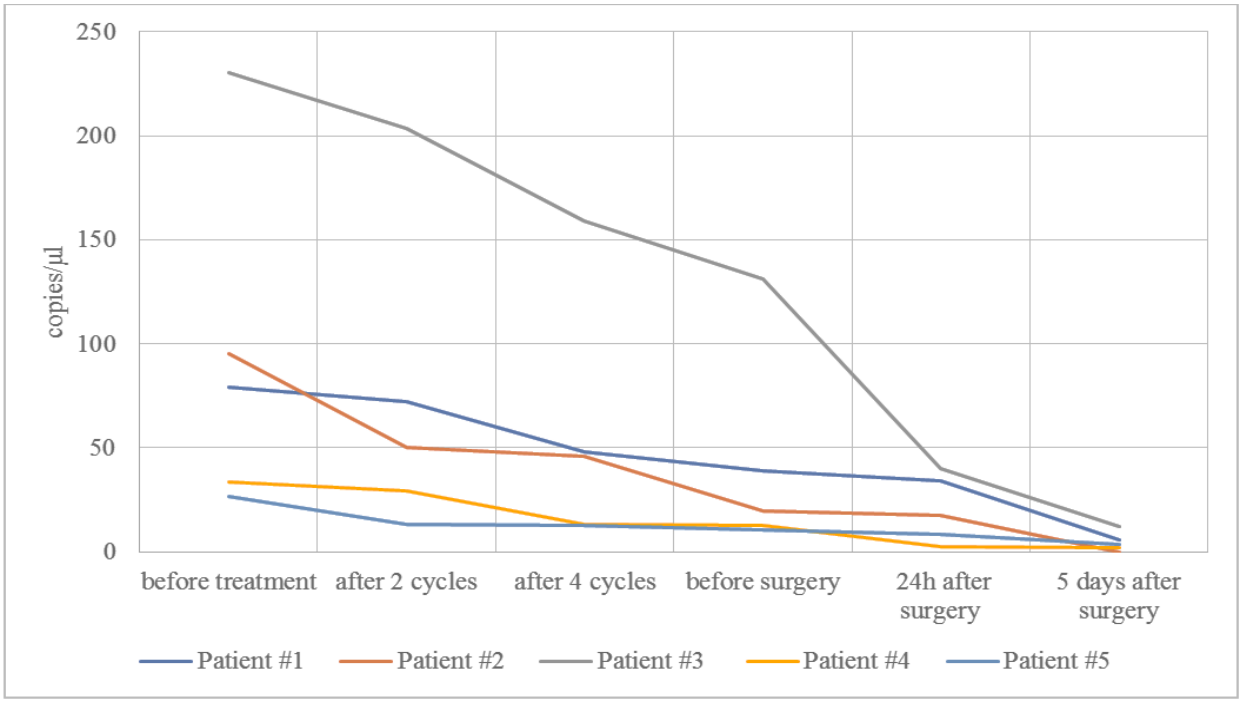


Fig. 5. *Dynamics of quantitative changes in PIK3CA-mutant circulating tumor DNA in patients with breast cancer studied during treatment.*

Discussion

The "gold standard" for cancer diagnosis is morphological verification of the tumor. However, in some cases, the establishment of a histotype of the neoplasm is not possible: general contraindications (serious condition of the patient), technical obstacles (lack of appropriate equipment or surgical equipment), lack of a primary focus with low tumor differentiation, patients refusing invasive

traumatic procedures, and others. Since the growth and development of a tumor is accompanied by its partial destruction, fragments of nucleic acids that can be recognized by typical mutations of oncogenes are released into the circulatory system [27-30]. Liquid biopsy is characterized by its potential ability to detect mutations in tumor DNA in a blood sample, which makes it possible to obtain a simple, non-invasive and quantitatively measurable method for use in a clinical setting [31].

Today, PIK3CA is one of the most frequently mutated oncogenes in breast cancer - 25–40% of cases [32]. The frequency of occurrence of this mutation differs significantly with different locations of the tumors, for example, a high frequency is typical for endometrial cancer, cervical cancer - 17-37%, and low - for cancer of the lung, thyroid gland - 4-5% [33]. In our study, the frequency of the identified PIK3CA mutation in samples of patients with locally advanced breast cancer was 17.24%, which was due to the search for only one “hotspot” variant of the mutation (H1047R). The mutation was predominantly associated with ER-positive and Her2/neu -negative tumor phenotypes. Similar data confirming the correlation between the mutational status of PIK3CA and hormone receptors-positive tumors were obtained by other researchers [34,35].

The average number of PIK3CA mutations at the screening stage for mutant and wild type alleles in biopsy samples averaged 396.4 and 7457.98 copies / microliter, in plasma samples - 92.9 and 8054.6 copies / microliter, respectively. The accuracy and reliability of the method for determining PIK3CA-mutant ctDNA using the digital PCR method is confirmed by the coincidence of the detected mutations in the samples of tumor tissue and ctDNA of blood plasma. This non-invasive manipulation can be repeated during treatment through reliable, reproducible and automated methods that can detect genomic changes with high sensitivity, which makes “liquid biopsy” an attractive concept in oncology [36–38].

During all cycles of neoadjuvant therapy and after surgery, there was a decrease in the number of ctDNA in plasma, and the lowest concentration was observed on the 1st and 5th day after the operation. In one patient in a plasma sample taken on the 5th day after surgery, the PIK3CA mutant ctDNA was completely absent. Our data on changes in the amount of PIK3CA mutant ctDNA during various stages of treatment help to track the dynamics of the tumor process comparable with the results of CT studies. This indicates the potential for detecting ctDNA as a marker for non-invasive monitoring of the malignant process and response to treatment in patients with locally advanced breast cancer. It should be noted that a high degree of concordance between the detection of PIK3CA mutations in tumor tissue and in the corresponding ctDNA was also found in other studies. That is why the PIK3CA mutant ctDNA can be used as an early marker for assessing the response to breast cancer treatment in addition to methods of visualization [31,39-41]. The dynamics of changes in the number of PIK3CA mutant ctDNA in serial plasma samples studied by digital PCR reflects the tumor burden and response to treatment and is comparable with computed tomography data.

It is expected that the study of ctDNA in breast cancer patients using digital PCR can become an additional diagnostic and prognostic marker that will not only monitor the effectiveness of the treatment, but will also serve as a reliable method for early detection of relapse of the disease, and will allow the diagnosis of malignant tumors of different localization at the earliest stage of development, individualize therapy, reduce the cost of the healthcare system for treatment and significantly increase the overall survival of patients.

Data regarding disease-free survival and overall survival of patients with PIK3CA-mutant breast cancer, compared with patients without detected PIK3CA-mutant ctDNA, will be presented in subsequent scientific papers. Further collection and analysis of samples is planned, as well as the inclusion of more patients in the study.

Conclusions

1. A mutation in the PIK3CA gene was found in 17.24% (5 samples 29 cases) of patients with locally advanced breast cancer. All cases of mutation were confirmed both in the tumor tissue and ctDNA in plasma.

2. For PIK3CA-mutant breast cancer, ER-positive (80%) and Her2/neu-negative (80%) tumor phenotypes are characteristic.

3. The use of a non-invasive study of PIK3CA-mutant ctDNA in locally advanced breast cancer can provide important information on the dynamics of the malignant process and help evaluate the response to treatment in such patients.

Conflict of interest

The authors declare that they have no competing interests.

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PIK3CA-МУТАНТНАЯ ЦИРКУЛИРУЮЩАЯ ОПУХОЛЕВАЯ ДНК У ПАЦИЕНТОВ С РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Резюме. При проведении исследования методом цифровой ПЦР были изучены образцы ткани и плазмы крови пациентов с местно-распространенным раком молочной железы на предмет наличия соматической мутации PIK3CA в биопсийном материале и циркулирующей опухолевой ДНК. Наличие мутации гена PIK3CA выявлено 17.24% (5/29) пациентов как в биопсийном материале, так и плазме крови. Исследования серийных образцов плазмы пациентов позволило выявить изменения количества циркулирующей опухолевой ДНК на протяжении всех этапов лечения, которые отражали динамику опухолевого процесса. Для PIK3CA-мутантного рака молочной железы характерными являются ER-положительные и Her2/neu-отрицательные фенотипы опухоли.

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