Bulgarian Chemical Communications, Volume 55, Special Issue C (pp. 87–93) 2023 DOI: 10.34049/bcc.55.C.0010

Application of liquid biopsy in patients with breast cancer

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Received: November 2023; Revised: December 2023

Breast cancer in women is the leading cause of global malignancy incidence for 2020 with an estimated 2.3 million new cases. Among women, breast carcinoma accounts for 1 in 4 cancer cases and 1 in 6 deaths. Projections indicate that the incidence of breast cancer will reach approximately 3.2 million per year by 2050.

The aim of our investigation is to develop and implement a modern algorithm for non-invasive monitoring of therapeutic response and minimal residual disease by liquid biopsy in patients with breast cancer. Liquid biopsy was studied in 50 breast cancer patients (26 in adjuvant and 24 in metastatic stage). ddPCR was performed to detect and quantify PIK3CA mutations. Patients testing positive for PIK3CA mutations underwent serial monitoring of their ctDNA.

PIK3CA mutations were identified in 8 (16%) of patients. On serial follow-up, five of the patients showed an increase in the amount of the mutation, which corresponded with poor response to treatment and fatal outcome. Conversely, three patients showed a decrease in the amount of the mutation, which was associated with a good response to treatment.

Liquid biopsy is an alternative for making a diagnosis for tumors in which it is difficult to conduct repeated invasive examinations. ctDNA is a non-invasive method to monitor tumor evolution, treatment response and assess patient prognosis. Serial ctDNA monitoring can help to predict relapse and to personalize therapy.

Keywords: breast cancer, liquid biopsy, ctDNA.

INTRODUCTION

The incidence of new cancer patients is increasing every year. Statistics show that by 2030, carcinomas will be the leading cause of death and the main barrier to increasing life expectancy in every country of the world in the 21st century. The reasons are diverse and include both population aging and population growth [1]. The main risk factors are associated with socio-economic development [2]. Breast cancer is the most commonly diagnosed cancer. Breast cancer is the fifth leading cause of cancer death in the world with 685,000 deaths. In countries with an average life expectancy of over 70 years, approximately 1 in 8 women will be diagnosed with breast cancer, 70% of cases occurring after the age of 60 [3].

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The increased incidence rates in countries with a higher HDI (Human Development Index) index can be explained by some reproductive and hormonal risk factors (early age of menarche, later onset of menopause, advanced age at first birth, younger number of children, less breastfeeding, menopausal hormone replacement therapy, oral contraceptive use), lifestyle risk factors (alcohol intake, overweight, lack of physical activity) as well as better diagnosis thanks to of mammographic screening [4].

Estimates indicate that the incidence of breast cancer will reach approximately 3.2 million annually by 2050. These figures reflect the impact on society worldwide and emphasize the need for timely preventive measures. Breast cancer screening programs aim to reduce mortality through early detection and effective treatment [5].

Breast cancer is a heterogeneous disease in terms of its etiology and pathological characteristics, in some cases it progresses slowly and has an excellent prognosis, and in others it has an aggressive course

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and an unfavorable outcome. In 63% of cases, the diagnosis is made in a localized stage, 28% in locally advanced, only in 5–6% in metastatic [6]. The overall five-year survival rate for breast cancer in the USA is 89.9%, for Europe – 86.6%, and in Bulgaria – 72.8%.

The different variants of this neoplasm are distinguished by variable histopathological and biological characteristics, varied response to the applied systemic therapies and different outcome of the disease.

The long-term prognosis for early-stage patients is good, but some tumors recur years after initial therapy is completed. Tumor heterogeneity and clonal evolution can be the reason for the development of distant metastases and resistance to the current treatment. These are also the leading causes of death.

Imaging studies and pathological analysis are leading in making the initial diagnosis, but they have their limitations in monitoring the effect of treatment, detection of residual disease and disease progression. Liquid biopsy is increasingly used as a non-invasive method for monitoring patients with oncological diseases. Detection of ctDNA in plasma can be used to non-invasively scan tumor genomes and determine tumor burden. Liquid biopsy allows detection of genomic alterations, therapeutic monitoring with early detection of resistance, and potential detection of disease progression prior to clinical and radiological confirmation.

MATERIALS AND METHODS

For the period 2020–2022 are tested patients with newly diagnosed carcinoma of the mammary gland treated at the Medical Oncology Clinic of UMHAT "Sv. Georgi", Plovdiv. 50 patients with breast cancer were monitored – 26 in the adjuvant stage and 24 in the metastatic stage. Patients were followed up before starting systemic treatment (baseline), at 1, 3 and 6 months by liquid biopsy for quantification of cfDNA. Genome sequencing was performed and somatic mutations were monitored by ddPCR.

Sequencing libraries were generated according to the instructions in the TruSight Tumor 15 Reference Guide. The assay requires a minimum of 10 ng of input DNA for each of 2 oligo pools (pool A and pool B), which are pooled and sequenced together after a library amplification step.

Libraries from plasma cfDNA were sequenced on a MiSeq® sequencing system with paired-end configuration (2×151 bp).

Illumina® TruSight® Tumor Protocol 15 describes a PCR-based multiplex method for preparing sequencing libraries from DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples. The reagents in the TruSight Tumor 15 kit allow the preparation of up to 48 indexed libraries paired from 24 DNA samples. The kit is optimized to provide amplicon coverage of 15 genes for high-sensitivity analysis of low-frequency somatic variants from FFPE samples of solid tumors. These genes and gene regions include single nucleotide variants (SNVs), insertions, deletions (indels) and amplifications that are associated with cancer.

The assay contains two separate sets of labeled oligonucleotide primers. These pools are used in multiplex PCR to amplify regions of DNA extracted from FFPE samples for specific purposes. Using adapters provided in the kit, libraries are indexed, further amplified, and then mixed in one tube in preparation for a paired-end sequencing run. After sequencing is complete, the analysis report provides a specific set of single nucleotide variants (SNVs) and small insertions, deletions, and amplifications associated with solid tumors.

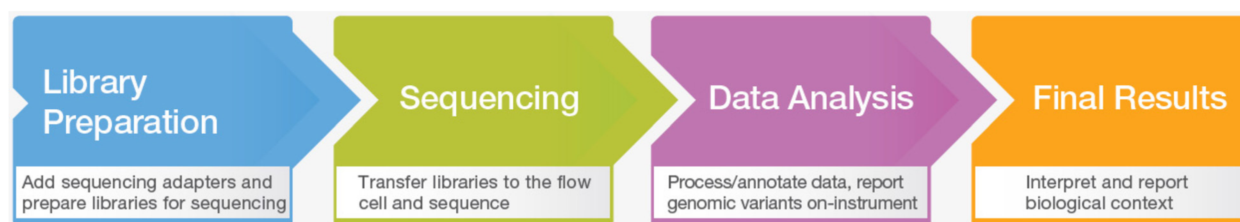

Fig. 1. MiSeq System Workflow – The MiSeq System's simplified workflow enables rapid NGS performance. Libraries can be prepared with any compatible library preparation kit. The sequencing time of five and a half hours included cluster generation, sequencing, and base counting with dual surface scan quality assessment for 2×25 base pairs running on a MiSeq system with MiSeq management software.

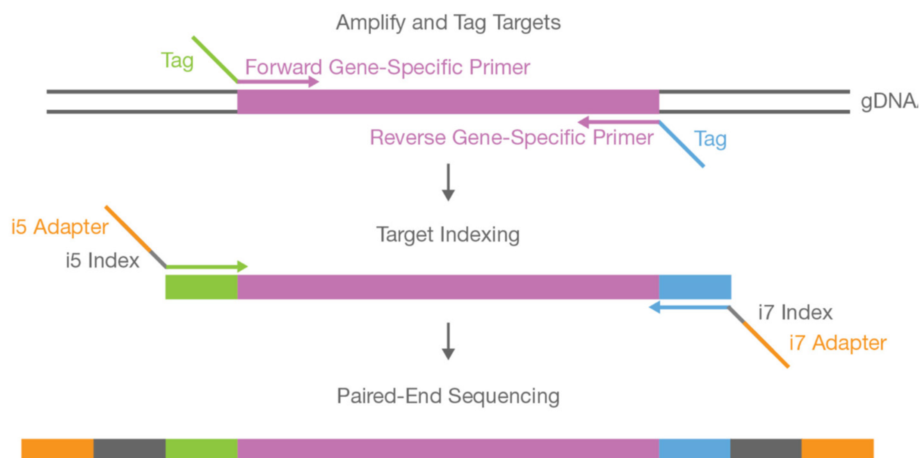

Fig. 2. How the analysis works.

DNA sequencing is the first and most basic information that can be obtained about a gene. This requires an automated and rapid system with a wide variety of applications, capable of de novo sequencing, resequencing (mutational profiling), microsatellite analyses, MLPA, AFLP, LOH, MLST, and SNP validation. (3500DX Series Genetic Analyzers (8-capillary sequencer/ GenomeLab™ GeXP Genetic Analysis System) with in vitro diagnostic capability)

Sequencing the genes responsible for the disease provides information in several directions:

1. Creating a personalized treatment plan for the disease based on the mutant gene and/or genes causing the disease, for example, sequencing the coding sequences of genes associated with breast cancer. The method is recommended when the patient has an unknown mutational status and does not belong to an ethnic group with characteristic mutations. Sequencing analysis revealed small deletions, insertions, missense, nonsense and splice site mutations.

2. The collected information from the group of patients with proven disease allows mapping of the most frequent mutations for the Bulgarian population, which allows the development of a faster and cheaper screening method based on DNA (free-cell DNA in tumors) and digital multiplex PCR, with specific primers. In this way, the patient will be diagnosed more quickly with the subsequent appointment of treatment, without the need to sequence the gene or genes. If a stretch of DNA containing a mutation has a known sequence, this can be used for clinical research. For this purpose, an oligonucleotide sample complementary to the region of interest with a mutation is synthesized. The sample will only bind complementary to DNA obtained from an individual with this mutation. The sample can be used as a PCR primer. If the DNA is amplified by PCR, the primer will not bind to normal DNA and there will be no amplification. Binding the primer to DNA from a patient and amplifying their DNA will indicate that the template DNA from that individual contains the mutation.

3. Isolation of cfDNA and monitoring of patients with proven somatic mutations by quantitative measurement of cfDNA by ddPCR.

MRD monitoring was performed by absolute quantitative (number of molecules per microliter) measurement of the established somatic mutation by means of digital real time PCR. Digital droplet PCR is a PCR technology that enables accurate absolute quantification of target molecules with a very high degree of sensitivity. In essence, the method combines the simplicity of traditional PCR and the functions of quantitative real time quantitative PCR (qRT-PCR). Unlike qRT-PCR, quantification is absolute and no calibration standards are used, making the process faster, more accurate and reproducible. The basic principle involves extreme dilution and separation of the sample into millions of individual units/droplets that ideally contain or do not contain the desired molecule. Each droplet contains all the reagents required for a PCR reaction and basically functions as a micro-PCR reactor. If the droplet contains the molecule of interest, PCR amplification gives a positive signal. If not - no signal. If the number of individual droplets is known, the initial amount of target molecules can be estimated from knowing the total number of positive and negative signals.

Because each droplet encapsulates a single molecule, researchers can quickly determine the absolute number of droplets containing a specific target DNA and compare the number to that of droplets with wild-type DNA. The Raindrop Digital Droplet PCR System shifts the current paradigm in digital droplet PCR (ddPCR) reaction from one marker-one color to two colors of different intensities per marker allowing multiplexing of targets up to 10 markers in one reaction.

Digital genomic technologies (Digital PCR (droplet)) offer higher sensitivity than massively parallel sequencing technologies and have been used as a method to validate results and quantify ctDNA. It is more cost-effective and faster methods in which a plasma ctDNA sample of patients can be used to non-invasively scan for tumor genes for breast cancer.

A droplet generator was used to divide the reaction mixture into about 20,000 nanoliter-sized droplets. Samples were subjected to polymerase chain reaction on a thermal cycler, droplets from each sample were analyzed on a dedicated QX200 droplet reader. The droplets pass sequentially through a two-color optical detection system. PCR-positive and PCR-negative droplets are counted, providing absolute quantification of the DNA of interest.

In our patients it was done ddPCR to detect and quantify PIK3CA mutations. Patients testing positive for PIK3CA mutations underwent serial monitoring of their ctDNA.

RESULTS

Patient characteristics

The clinico-pathological characteristics of the tumors of the 50 patients examined are presented in Figure 3: with luminal type A there are 29 patients (58%), with luminal type B there are 6 patients (12%), with HER2-positive BC there are 11 patients (22%) and with TNBC were 4 patients (8%).

All patients underwent genomic sequencing after surgery and before starting chemotherapy (Table 2).

The frequency of the studied genes corresponds to that described in the literature. A total of 8 (16%) patients had a PIK3CA mutation in their primary tumor (Fig. 4).

PIK3CA mutations (H1047R, E545K, E542K, N345K, and H1047L) were analyzed with the QX200 Droplet Digital PCR System (Bio-Rad Laboratories) (Fig. 5). 5 patients had mutations in exon

Table 1. Clinical and laboratory characteristics of the patients

Parameters	Patients n (%)
Family history	20 (40%)
BMI ≥ 35, n (%)	24 (48%)
Hgb < 100 g/l, n (%)	17 (34%)
WBC ≥ 10.5 G/l, n (%)	11 (22%)
PLT ≥ 350 G/l, n (%)	14 (28%)
Ca 15-3 ≥ 25 U/l, n (%)	26 (52%)
CEA ≥ 3 ng/mL, n (%)	18 (36%)

Table 2. Gene list in TruSight Tumor 15 kit

TruSight Tumor 15 Gene List		
AKT1	GNA11	NRAS
BRAF	GNAQ	PDGFRA
EGFR	KIT	PIK3CA
ERBB2	KRAS	RET
FOXL2	MET	TP53

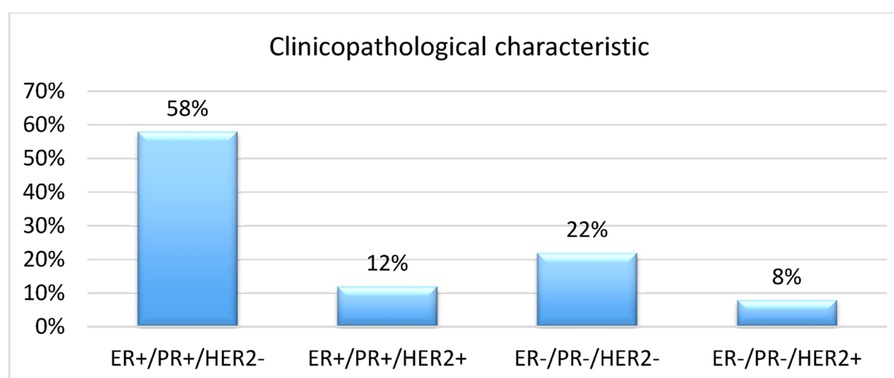

Fig. 3. Distribution of patients with breast cancer according to IHC.

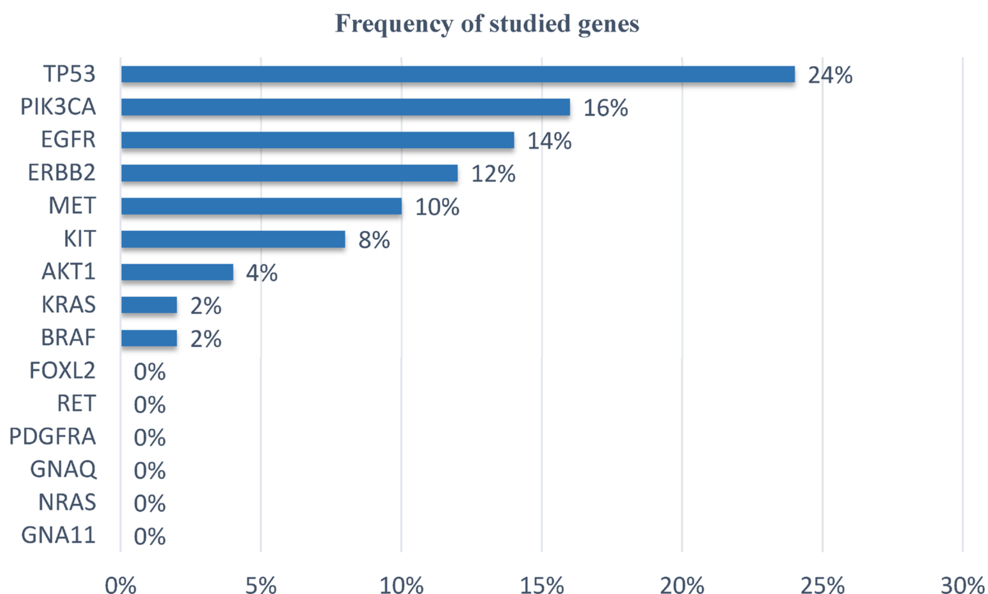

Fig. 4. Frequency of studied genes in breast cancer patients.

9 (E542K and E545K) and 2 had mutations in exon 20 (H1047L and H1047R). In 1 patient was found a mutation in N345K.

Of these 8 patients with PIK3CA mutations, 3 had luminal type A tumor, 2 had luminal type B, 2 had HER2-positive tumors, and 1 had TNBC. 5 of the patients received 6 courses of chemotherapy, and 3 of them received endocrine therapy. In serial follow-up, five of the patients showed an increase in the amount of the mutation, which corresponded with a poor response to the ongoing treatment and a fatal outcome. In contrast, 3 patients showed a decrease in the amount of the mutation, which was associated with a good response to treatment.

DISCUSSION

Clinicopathological characterization of breast cancer was determined by estrogen receptor (ER),

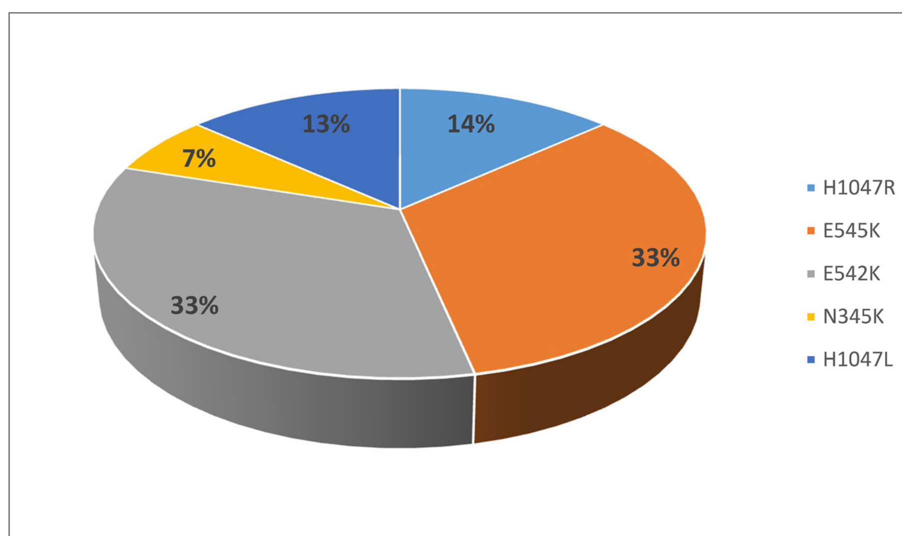

Fig. 5. Frequency of the most common mutations in PIK3CA examined by ddPCR.

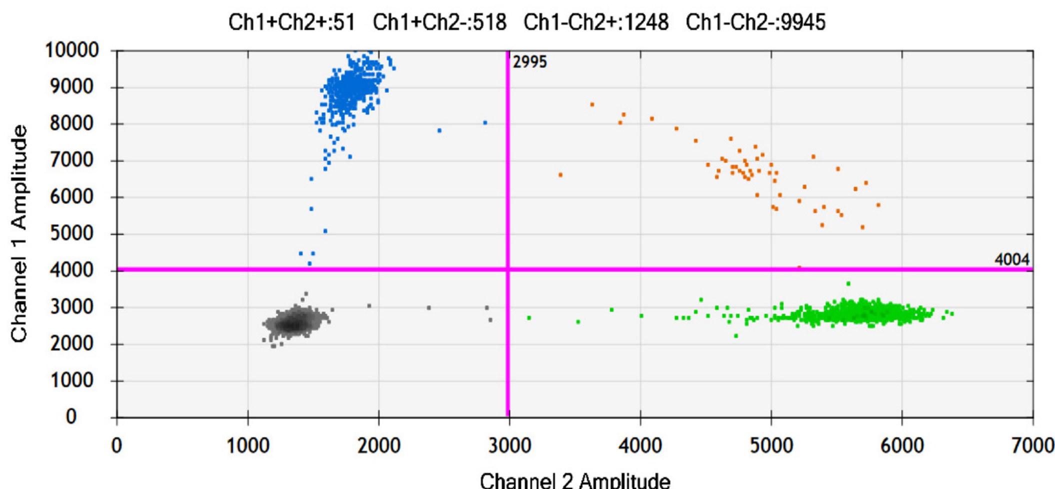

Fig. 6. Results from ddPCR- black droplets are empty droplets, green droplets are with wild type mutations, orange droplets are droplets having signals for both wild type and mutant type mutations, and blue droplets are for mutant alleles only.

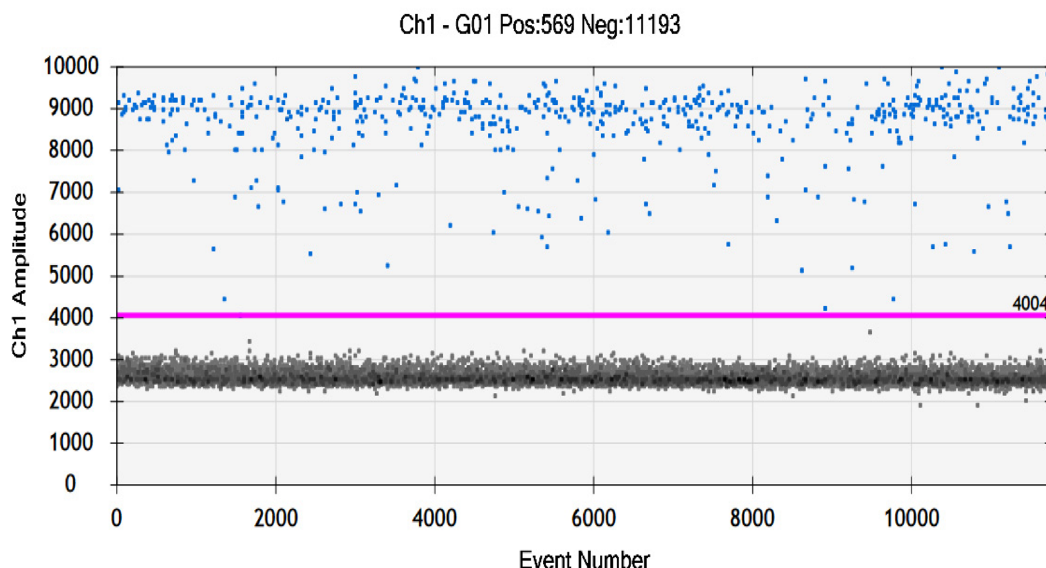

Fig. 7. Results from ddPCR-mutant alleles.

progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2) and Ki67 status. Depending on this, we distinguish 4 immunohistochemical (IHC) tumor subtypes: luminal A, luminal B, HER2-positive type and triple negative (TN) type. Therapeutic behavior is determined depending on the specific subtype. However, genetic aberrations occurring in the tumor can significantly alter the effect of the treatment being administered [7].

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) the gene encoding phosphatidylinositol-3-kinase (PI3K) catalytic subunit p110-alpha is commonly mutated in breast cancer (21–35%) and is associated with progression of the tumor. This leads to hyperactivation of the PI3K/AKT/mTOR pathway, which plays a key role in several cellular processes related to oncogenesis—migration, metabolism, cell growth, and proliferation [8].

The most frequently encountered point mutations are E545K, E542K and H1047R/L, they are responsible for 70–80% of all PIK3CA mutations. The frequency is highest in luminal type and HER2-

positive breast cancer, lower in triple-negative breast cancer [9]. The presence of the PIK3CA mutation is also associated with resistance to targeted therapy with trastuzumab [10]. ER-positive patients who have PIK3CA mutations show better results from treatment with aromatase inhibitors. PIK3CA mutations are potential targets and predictive markers for the efficacy of novel molecularly targeted agents [11]. Alpelisib is a PI3Kα tyrosine kinase inhibitor that was tested in the SOLAR-1 study. Patients in this trial who had received prior endocrine therapy received either a combination of alpelisib/fulvestrant (a synthetic estrogen receptor antagonist) or placebo/fulvestrant. Mutational status was found to be a predictive response factor for progression-free survival (PFS). Final results for overall survival (OS) demonstrated a 7.9 month numerical improvement for this group of patients [12]. Based on data from this clinical trial, the FDA approved in May 2019 the combination of alpelisib with fulvestrant in postmenopausal women and men with HR+/HER2−, PIK3CA-mutated, advanced, or metastatic breast cancer after progression during or after treatment with endocrine-based regimen. In 2020, the European Medicines Agency (EMA) in turn granted a marketing authorization for alpelisib.

Therefore, the detection of PIK3CA mutations has a crucial role in identifying patients who will benefit from treatment with alpelisib. There is currently no consensus on the best analytical method (liquid biopsy or tissue) or the best type of biopsy (primary tumor or metastases).

The presence of this tumor heterogeneity makes its treatment challenging. Furthermore, there is a high frequency of discordance in PIK3CA mutations between primary, locally advanced, breast cancer tumors and metastatic stage carcinomas. Adequate samples for tissue analysis are not always available or accessible. Even when available, tissue obtained during primary surgical resection or biopsy may not reflect the current molecular characteristics of the tumor [13, 14, 15]. Liquid biopsy analyzes plasma-derived circulating tumor DNA (ctDNA) and offers less invasive real-time information on the genetic changes that have occurred [16].

CONCLUSIONS

Liquid biopsy is an alternative for the diagnosis of tumors in which it is difficult or impossible to perform re-invasive examinations, as well as for restaging and molecular analysis of metastases. According to the ESMO recommendations, cfDNA evaluation is considered a good alternative for metastatic tumor analysis and is an option to detect patients suitable for targeted therapy with alpelisib. The high sensitivity, efficiency and low cost of multiplex dPCR assays make them suitable for qualitative and quantitative clinical detection of PIK3CA mutations in plasma. Liquid biopsy could be a screening method for populations at increased risk of developing malignancy, thus reducing the side effects of therapies and healthcare costs.

Acknowledgments: *Project BG05M2OP001-1.002-0005 – Competence Center; „Personalized Innovative Medicine (PERIMED)“, financed by Operational Program „Science and Education for Smart Growth“, EU, ESIF.*

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