Molecular analysis of circulating tumor DNA from breast cancer patients before and after surgery and following adjuvant chemotherapy

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Abstract. The primary aim of the present study is to provide a complex molecular profile of tumors using liquid biopsy and to monitor profile changes over time in association with surgery and administered adjuvant therapy. Our secondary aim was to compare the liquid biopsy profile with the tissue biopsy and assess concordance. A total of 27 samples of circulating tumor DNA (ctDNA) collected from 9 breast cancer patients at three different time points and their matched formalin-fixed and paraffin-embedded (FFPE) samples of primary tumor were analyzed with targeted next-generation sequencing. Somatic pathogenic variants were detected before surgery in samples from 5 patients (55.6%). The most frequently mutated genes were phosphatase and tensin homolog (4/9, 44.4%) and tumor protein 53 (4/9, 44.4%). Serial sampling of ctDNA enabled the detection of more variants compared with single-time tissue primary tumor biopsy. There were 17 ctDNA variants across all samples, but only 6 FFPE variants across all patients. In addition, the concordance between ctDNA and FFPE DNA was determined in only 1 patient, and this was connected with higher variant allele frequency. The findings of the present study suggest that liquid biopsy and tissue biopsy may be used as complementary analyses to adequately capture all tumor variants.

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Introduction

Breast cancer (BC) is the most commonly diagnosed carcinoma in women, accounting for almost 1 in 4 female cancers, and it is the leading cause of cancer-related mortality in women globally (1). BC is a complex heterogenous disease defined by its diverse aggressive behavior, potential to metastasize and therapeutic gene targets. The primary tumor generally contains combined diverse clonal populations of cells; therefore, invasive biopsy of the solid tumor may be biased and provide only an incomplete molecular profile of the analyzed tumor (2).

Circulating tumor DNA (ctDNA) has the potential to capture complete information on genetic alterations, including the somatic single-nucleotide variations present in the tumor and its metastases, and also to longitudinally monitor these mutations throughout the course of patient treatment (3,4). In addition, ctDNA is considered to be released from apoptotic and necrotic tumor cells, and its blood circulation half-life ranges from minutes to several hours, which makes it a dynamic, almost real-time, biomarker for cancer monitoring (5,6). The detection of ctDNA, however, presents a serious challenge, as ctDNA is highly fragmented to on average 170 bp in length (7,8). ctDNA may occasionally be present only as a minimal fraction in the circulation compared with the wild-type cell-free DNA (cfDNA) released from normal cells (4). In healthy individuals, cfDNA originates from hematopoietic cells (9,10). However, ctDNA of cancer patients is tissue-specific, as the majority of their ctDNA comes from the tumor tissue of origin (9-11). The ratio of ctDNA to cfDNA depends on tumor and immunological factors, tumor burden and progression, and blood clearance mechanisms (12,13).

Targeted next-generation sequencing (NGS), which generates sufficiently high coverage over a certain region, is therefore required, and ctDNA molecular analysis and identification of these low-frequency variants is important for detection of cancer in its early stages and appropriate diagnosis. Moreover, ctDNA analysis in the clinical setting may

guide decision-making and potentially facilitate personalized cancer care (8,14,15).

In BC, recent studies have indicated the versatile role of ctDNA analysis in determining the genomic profile of tumors (16-19), to sensitively and specifically identify preclinical metastases and predict a relapse ahead of its diagnosis (20-22), and to asses resistance to targeted therapy or to uncover tumor heterogeneity (23).

The primary aim of the present study was to provide a complex tumor molecular profile with liquid biopsy and to monitor its changes over time in association with surgery and adjuvant therapy. The secondary aim was to compare this liquid biopsy profile with the tissue biopsy and to assess concordance.

Materials and methods

Patients. Patients with invasive BC were treated at the Clinic of Gynecology and Obstetrics at the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava (JFM CU) and University Hospital in Martin (UHM). All subjects provided their written informed consent and the study was conducted in accordance with the Declaration of Helsinki and following the protocol approved by the Ethics Committee of JFM CU (IORG0004721) under project identification code EC 1525/2014. The patient data were collected retrospectively from medical records.

Blood sample processing. Serial peripheral whole-blood samples were drawn from 9 patients at three different timepoints: Before surgery, ~2 days after surgery and again 7 months following adjuvant chemotherapy. Briefly, 10 ml blood samples (n=27) were collected in K_3 EDTA tubes (Sarstedt AG & Co.) and processed within 2 h. Plasma samples were separated by two-step centrifugation at 2,200 x g for 8 min at 4˚C and 20,000 x g for 8 min at 4˚C. The plasma samples were then stored at -80˚C until DNA extraction.

DNA extraction and quantification. ctDNA was isolated from 3 ml plasma by the commercially available QIAamp DSP Virus kit (Qiagen GmbH), according to the manufacturer's instructions, and with the QIAvac 24 Plus vacuum manifold (Qiagen GmbH). The plasma samples were lysed, ctDNA was bound to a silica-based membrane, washed with ethanol-containing buffers, and waste was drained by vacuum pressure. The ctDNA was eluted in 30 μl sterile distilled water and ctDNA concentration was quantified with the Qubit dsDNA HS Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) on the Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted ctDNA was then concentrated using the CentriVap Centrifugal concentrator 78100 (Labconco) at 37˚C for 90 min and ctDNA was re-suspended in 30 μl sterile distilled water. Finally, the resultant ctDNA samples were stored at -20˚C until analysis.

DNA was also isolated from 9 formalin-fixed and paraffin-embedded (FFPE) tissue samples obtained from the Department of Pathological Anatomy of JFM CU and UHM. The commercially available BlackPREP FFPE DNA kit (Analytic Jena AG) was used according to the manufacturer's instructions. In brief, FFPE slides were lysed and shaken at 65˚C for 1 h, followed by 1 h at 90˚C. DNA was bound to a spin filter membrane and washed with ethanol-based buffers and then eluted in 50 μl elution buffer. The DNA concentration was quantified with the Qubit dsDNA BR Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) on the Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.SA) and DNA samples were stored at -20˚C until analysis.

DNA library preparation. DNA libraries (n=35) for NGS were prepared using the TruSight Tumor 26 kit (Illumina, Inc.) according to the manufacturer's instructions. Briefly, the quality and the amplification potential of DNA extracted from FFPE samples were determined by quantitative (q)PCR analysis using the KAPA SYBR FAST qPCR Universal Master Mix kit (Kapa Biosystems; Roche Diagnostics). A ΔCq value was calculated for each sample by comparing the amplification potential of FFPE DNA with the amplification potential of reference QCT DNA provided in the kit. DNA libraries (n=8) were prepared with either 20 μl of neat FFPE DNA or 20 μl diluted FFPE DNA depending on the ΔCq value of the corresponding sample (Table I). Although sample 7.2 had low concentration, the DNA library could be prepared; however, sample 9.2 had almost no DNA detected on Qubit assay and was therefore excluded from further analysis. The ctDNA sample quality was not investigated, and the DNA libraries (n=27) were prepared with 20 μl neat ctDNA.

FPA and FPB, two separate complementary oligonucleotide pools, were hybridized to DNA samples overnight. This process resulted in generation of A and B complementary DNA libraries for every DNA sample by targeting the positive and the negative DNA strands of the same region. The oligonucleotide pools are specific to the targeted regions of interest in 174 amplicons of 26 genes (Table II). Unbound oligonucleotides were removed, and bound oligonucleotides were extended and ligated. PCR amplification with indices and adapters added to extension-ligation products was then performed, and the libraries were purified using AMPure XP magnetic beads (Beckman Coulter, Inc.). The libraries were quantified using Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.) on the 2100 Bioanalyzer (Agilent Technologies, Inc.) according to the manufacturer's instructions, and DNA library concentrations were then quantified by the Qubit dsDNA HS Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) on the Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.).

DNA sequencing and data analysis. Targeted amplicon sequencing was performed on MiSeq (Illumina, Inc.), with MiSeq Control Software version 2.6.2, according to the manufacturer's instructions. Only four library A and four library B samples could be sequenced per run. In brief, the libraries were normalized to 4 nM and pooled into a single tube. Then, 600 μl of pooled libraries were loaded into a MiSeq reagent cartridge v2 (300 cycles) and sequenced on flow cell using 150 bp paired-end sequencing protocol. The raw sequence data were de-multiplexed and converted into FASTQ files using MiSeq Reporter version 2.6. The sequencing reads were aligned to the Genome Reference Consortium Human Build 37 (GRCh37)/Human Genome version 19 (hg19). Variants were called by Somatic Variant Caller Version 3.2.1 and written

Table I. ΔCq values of formalin-fixed and paraffin-embedded DNA samples with the corresponding dilution.

Sample	ΔCq	Dilution
1.2	0.98	2x
2.2	3.63	No dilution
3.2	3.42	No dilution
4.2	-0.16	4x
5.2	2.17	No dilution
6.2	2.45	No dilution
7.2	Low DNA concentration	No dilution
8.2	0.17	4x
9.2	No DNA	

Table II. Genes included in TruSight Tumor 26 kit (Illumina, Inc.).

AKT1	*ERBB2*	*KRAS*	*PTEN*
ALK	*FBXW7*	*MAP2K1*	*SMAD4*
APC	*FGFR2*	*MET*	*SRC*
BRAF	*FOXL2*	*MSH6*	*STK11*
CDH1	*GNAQ*	*NRAS*	*TP53*
CTNNB1	*GNAS*	*PDGFRA*	
EGFR	*KIT*	*PIK3CA*	

into VCF files. Variants with variant allele frequency (VAF) <3.0%, quality score <30, low depth of coverage or significant strand bias were filtered out, and strand bias filler removed sequencing artifacts that were asymmetrically represented only in one strand. The resulting variants were annotated using the open source Variant Effect Predictor version 93 bioinformatics software tool (24). The classification of variants was based on VarSome v6.7, which uses the American College of Medical Genetics and Genomics guidelines (25,26). The yielded pathogenic variants were subsequently checked against the Catalogue of Somatic Mutations in Cancer (COSMIC) database version 88 for somatic categorization (27). Finally, variants of unknown significance or germline and benign variants were filtered out.

Results

Patient characteristics. A total of 9 female patients with histopathologically confirmed BC were analyzed. The mean age was 56.22±9.62 years and the histopathological characteristics of the patients are summarized in Table III. A complete list of the patients' characteristics is provided in Table SI. Most patients (55.6%) had invasive ductal carcinoma (IDC) and all tested samples were estrogen receptor (ER)-positive. Only 2 samples were human epidermal growth factor receptor 2 (HER-2)-positive and the HER-2 status of 2 patients was unavailable. The molecular subtypes were luminal A (44.4%) or luminal B (44.4%), and the molecular subtype of 1 patient was unknown due to missing data.

Table III. Histopathological characteristics of patients with BC.

Characteristics	N (%)
Histological type	
IDC	5 (55.6)
ILC	4 (44.4)
Histological grade	
G1	2 (22.2)
G2	4 (44.4)
G3	3 (33.3)
ER status	
Positive	9 (100.0)
Negative	0 (0.0)
PR status	
Positive	8 (88.9)
Negative	1 (11.1)
HER-2 status	
Positive	2 (22.2)
Negative	5 (55.6)
Unknown	2 (22.2)
Ki-67 status	
Low	5 (55.6)
High	4 (44.4)
Molecular subtype	
Luminal A	4 (44.4)
Luminal B	4 (44.4)
Unknown	1 (11.1)

BC, breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; Ki-67 proliferation index.

Molecular analysis of ctDNA and FFPE DNA. We monitored somatic variants with pathogenic effect in ctDNA from three different time points and FFPE samples in 9 patients with BC across 26 genes (Table IV). Each patient had at least one somatic pathogenic variant. The majority of somatic pathogenic variants were found in samples prior to surgery in 5 patients (55.6%). No somatic pathogenic variants were detected in the patients' follow-up samples, postoperatively or after adjuvant therapy. The patients had low-grade (G1 or G2) BC, and 4 had luminal A subtype.

By contrast, somatic pathogenic variants were detected only in postoperative samples in patients 4 and 5 (22.2%). The patients had higher-grade (G2 or G3) cancer, unknown HER-2 status and luminal B or unknown luminal type. Patients 2 and 6 had somatic pathogenic variants only in samples after chemotherapy (22.2%). These patients had luminal B, HER-2$^+$ and grade 3 BC.

No somatic pathogenic variants were detected in FFPE DNA samples of patients 3 and 6. Complete accord between ctDNA and FFPE DNA was observed only in patient 4 (11.1%).

Table IV. Overview of identified somatic pathogenic variants of genes in patients with BC.

	PreOp			PostOP			PostChem			FFPE		
Patients	Gene	Variant	Effect	Gene	Variant	Effect	Gene	Variant	Effect	Gene	Variant	Effect
1 (IDC, LA, G1)	*PTEN*	c.701G>A	Missense		Low-quality calls			No sample				
	TP53	c.770T>A	Missense									
	PIK3CA	c.3140A>T	Missense							*PIK3CA*	c.3140A>G	Missense
	APC	c.2639T>C	Missense									
2 (IDC, LB, HER-2+, G3)		Not detected			Not detected		*BRAF*	c.1397G>A	Missense			
										PTEN	c.428delC	Frameshift
3 (IDC, LB, G2)	*PTEN*	c.43A>G	Missense		Not detected			Not detected			Not detected	
		c.101C>A	Missense									
	APC	c.4319delC	Frameshift									
		c.4495G>T	Nonsense									
4 (ILC, LB, G3)[a]		Not detected		*CDH1*	c.1921C>T	Nonsense		Not detected		*CDH1*	c.1921C>T	Nonsense
5 (ILC, UNK, G2)[a]		Not detected		*TP53*	c.1101-2A>T	Splice acceptor		No sample				
										PIK3CA	c.3140A>G	Missense
6 (IDC, LB, HER-2+, G3)		Not detected			Not detected		*NRAS*	c.46A>G	Missense		Not detected	
							TP53	c.973G>T	Nonsense			
7 (IDC, LA, G1)	*PIK3CA*	c.3139C>T	Missense		Not detected			Not detected		*PIK3CA*	c.3140A>T	Missense
	BRAF	c.1799T>A	Missense									
8 (ILC, LA, G2)	*PTEN*	c.210-2A>G	Splice acceptor		Not detected			Not detected				
										KRAS	c.35G>T	Missense
9 (ILC, LA, G2)	*TP53*	c.1031T>C	Missense		Not detected			Not detected			No sample	

[a]HER-2 status is unknown. BC, breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; LA, luminal A; LB, luminal B; UNK, unknown; HER2, human epidermal growth factor receptor 2; G, grade; PreOp, before surgery; PostOp, after surgery; PostChem, after adjuvant chemotherapy; FFPE, formalin-fixed and paraffin-embedded tissues.

Although phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations in codon 1,047 were found in patient 1 and 7 ctDNA and FFPE DNA, the protein variants were different: p.His1047Leu in the ctDNA and p.His1047Arg in FFPE in patient 1, and p.His1047Tyr in the ctDNA and p.His1047Leu in FFPE in patient 7. In addition, while patient 6 displayed negative concordance with no somatic pathogenic variants detected in the ctDNA sample before surgery or the FFPE sample, two somatic pathogenic alterations were detected in the ctDNA sample after chemotherapy. Patient 9 FFPE sample was excluded from DNA library preparation due to insufficient DNA concentration. Two ctDNA libraries, post-chemotherapy ctDNA samples of patient 1 and 5, were not sequenced because of technical difficulties. Finally, the postoperative ctDNA sample of patient 1 generated only low-quality calls, which were filtered out.

Overall, 23 variants in 8 different genes were detected in the present study, and these were mostly missense mutations (15/23, 65.22%). The most common mutation type was single nucleotide substitution (21/23, 91.3%), and the most frequently mutated genes were phosphatase and tensin homolog (*PTEN*; 4/9, 44.4%), tumor protein 35 (*TP53*; 4/9, 44.4%) and *PIK3CA* (3/9, 33.3%; Table SII).

The mean depth of coverage per targeted region in the ctDNA samples was 12,926±8,120 (range, 2,261-29,762). The mean variant allele frequency (VAF) in ctDNA samples was 4.74±1.95% (range, 3.04-10.43%) (Table SIII). The mean depth of coverage per targeted region in FFPE DNA samples was 25,169±9,533 (range, 12,881-40,713). Finally, the mean VAF in FFPE DNA samples was 25.91±15.15% (range, 8.04-50.53%; Table SIV).

Discussion

In the present study, the molecular profile in serial ctDNA samples from BC patients was monitored and it was demonstrated that ctDNA sequencing provides dynamic information on somatic variants with pathogenic effects in tumors. The molecular analysis revealed that patients with lower-grade and luminal A BC subtype and 1 patient with low-grade luminal B subtype had somatic pathogenic variants detected only in their ctDNA prior to surgery. This correlates with a generally better prognosis of the luminal A subtype compared with other BC subtypes (28-30). Detection of somatic pathogenic variants in ctDNA only after surgery was associated with patients of unknown HER-2 status and luminal B, and unknown luminal type; but these patients had higher-grade (G2 or G3) cancer. While patients with luminal B, HER-2$^+$ and grade 3 had somatic pathogenic variants detected in ctDNA only after chemotherapy, restricted patient numbers, lack of characterization and impossibility of analyzing certain samples prevented significant conclusions from these results.

The number of our patients was restricted as only a limited number of BC patients admitted to the hospital was able to comply with the demanding serial sampling.

Another limitation of the present study was the exclusion of important BC genes, such as *BRCA1*, *BRCA2* and *ESR1*. We selected TruSight Tumor 26 kit with the panel of 26 oncogenes and tumor suppressor genes, which are the most frequently altered in solid cancers. This kit was designed to detect somatic variants in highly degraded and fragmented samples, such as FFPE slides, and it was inferred that it would also be suitable for fragmented ctDNA samples.

Although detecting somatic variants without matched normal tissue sample is possible, albeit difficult, VAF denoting the number of reads that support the mutated allele at a given locus can help distinguish somatic from germline variants in both tumor samples and ctDNA. In theory, the somatic variants should only be present in the tumor cells and, thus, give a low VAF in ctDNA, whereas the germline variants would be present in both the sample tumor and normal cells, resulting in a VAF of ~50% for heterozygous variants and ~100% for homozygous (31). In addition, the VAF of somatic variants in analyzed tissues depends on the numbers of tumor and normal cells, and this enables their possible detection at any level (32).

The variants herein were considered somatic if they were included in version 88 of the COSMIC database (27). While we were able to detect variants with low VAF in the ctDNA and FFPE samples, concordance between ctDNA and FFPE DNA was only determined in 1 patient. This patient had a higher VAF compared with other patients' detected variants, and the variant detected in the *CDH1* gene had both the highest VAF in ctDNA (10.43%) and FFPE DNA (50.53%). Consistently with these findings, Chae *et al* also recorded this connection between higher VAF and concordant mutations in ctDNA and tissue biopsy from a patient with BC (17).

Somatic *PTEN* mutations in BC are rare, with a reported frequency of 5-10% (33-35). The frequency of *PTEN* alterations in our patients was 44.4%. It is hypothesized that the difference may be caused by restricted number of our patients. There was no association between *PTEN* alterations and distinctive clinicopathological characteristics in our patients. However, there is no consistent evidence to prove the real prognostic role of *PTEN* mutations in BC due to the lack of reproducibility in studies (35). The frequency of *TP53* mutations in BC is ~40% (33,36). *TP53* mutations were detected in 44.4% of our patients. There was no association between *TP53* mutations and distinctive clinicopathological characteristics in our patients. However, several studies demonstrated that *TP53* mutations occur more frequently in HER-2-enriched tumors compared with luminal A or luminal B tumors (37,38). By contrast, high frequency of *PIK3CA* mutations (33.3%) in our patients was correlated with 30-40% prevalence of *PIK3CA* mutations in patients with BC (33,39). Our patients with *PIK3CA* had lower-grade tumors. The association of *PIK3CA* mutations and lower-grade ER$^+$ BC was shown in the analysis of pooled data by Zardavas *et al* (40).

Alborelli *et al* (41), analyzed cfDNA of 114 clinically healthy individuals for genetic alterations with a limit of detection as low as 0.08% of VAF. The majority of the subjects (84%) had no genetic alterations in cfDNA. However, the authors identified pathogenic cancer alterations in 4 healthy individuals. The alterations were detected in cancer hotspot variants, including *PIK3CA* p.His1047Arg. These individuals developed a benign neoplasm or invasive breast in the 10 years following blood collection. Therefore, the analysis of cfDNA in healthy subjects may be relevant to the early detection of cancer.

Serial sampling of ctDNA enabled detection of more variants than in one-time primary tumor tissue biopsy. The results determined 17 ctDNA variants in all samples, but

only 6 FFPE variants in all patients. The different number of detected variants and their diversity may be attributed to intratumor heterogeneity, where ctDNA may be released into the bloodstream from a completely unique cell subpopulation with mutations different to those present in the primary tumor (2). Moreover, alterations identified only in ctDNA may be missed by single-site tissue biopsy due to sampling different tumor areas and incomplete genetic profiling (42). Mutations with lower VAF identified only in FFPE samples may be unaccounted for in ctDNA analysis due to their under-representation in the circulation. Therefore, we suggest that liquid biopsy and tissue biopsy should be complementary analyses with combined capability of capturing all tumor variants.

The advantages of ctDNA analysis and the liquid biopsy were also demonstrated by Finzel *et al* (43), who studied data of 351 patients with stage IV solid tumors, including BC, focusing on the discordant gene mutations identified between FFPE blocks and ctDNA from blood samples. The authors detected different molecular alterations between tissue and liquid biopsies in 86% of patients. However, 42% of the mutations detected only in the liquid biopsy had clinical relevance in prediction of sensitivity or resistance to administered targeted therapies. The combined assessment of liquid and solid biopsies enables better characterization of tumor heterogeneity and important information for patient therapy (43).

Rodriguez *et al* analyzed *PIK3CA* and *TP53* mutations in matched ctDNA samples before biopsy and tumor biopsy samples in patients with early BC. Concordance between ctDNA mutations and biopsy mutations was observed in only 8/29 (27.6%) patients. All patients with concordant mutations had higher-grade disease. Somatic *PIK3CA* mutations were identified in 19/29 (65.5%) patients. *TP53* mutations were identified in 6/29 (20.7%), and 4 patients (13.8) had both *PIK3CA* and *TP53* mutations. Moreover, the authors identified 4 additional mutations in ctDNA, which were not detected in tissue biopsy. They proposed that ctDNA analysis could identify tumor heterogeneity, improve the diagnosis of early BC patients, and provide significant information (44).

Similar studies have been performed in ER^+ and triple-negative BC, but these studies used different methods, or mainly focused on comparing tissue biopsy results with the ctDNA detected in metastatic disease (16,45,46). In a prospective study, Beaver *et al* (16) analyzed 30 primary breast tumor samples for frequently occurring *PIK3CA* mutations. Therein, they used Sanger sequencing and matched pre- and postoperative plasma tumor DNA (ptDNA) from 29 patients with early-stage BC by droplet digital PCR (ddPCR) analysis. This ddPCR analysis verified all the mutations detected in the tumor by Sanger sequencing and identified five new mutations, with concordance between tumor and preoperative ptDNA observed in 14/15 *PIK3CA* mutations. A total of 10 patients were mutation-positive in preoperative ptDNA samples, and 5 of them were also mutation-positive in postoperative ptDNA. This study demonstrated precise mutation detection in tumor thorough ddPCR, and also that ptDNA can be identified in pre- and postoperative blood samples of patients with early-stage BC.

Furthermore, Chung *et al* (45) determined ctDNA genomic changes in patients with ER^+ metastatic BC (mBC) by hybrid capture-based genomic profiling. They analyzed time-matched ctDNA and tissue samples from 14 patients and 89% of mutations in tissues were also present in the in ctDNA, but mutations in *ESR1*, *TP53* and *PIK3CA* were detected only the in ctDNA samples. The authors therefore considered that this detection of extra mutations in ctDNA could support liquid biopsy use in detecting heterogenous metastatic sites in ER^+ mBC. Stover *et al* (46), conducted a retrospective study using cfDNA to determine somatic copy-number variations in primary and metastatic tumors and cfDNA tumor fractions in patients with triple-negative BC. Their work highlighted that cfDNA tumor fractions ≥10% were significantly associated with worse survival.

In conclusion, the results of the present study add to the mounting evidence supporting the utility of ctDNA as complementary analysis to tissue biopsy for thorough molecular profiling of tumor variants. Finally, it was demonstrated that next-generation ctDNA sequencing is useful for monitoring the changes in somatic variants with pathogenic effects in patients with BC, and this analysis can supplement the results of tumor tissue biopsy.

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Availability of data and materials

All data generated or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KZ, MJ and KD formulated and designed the study. ZL and MB collected the samples, obtained patients' written informed consents, and created patients' database. KZ and MJ analyzed the samples and carried out NGS analysis. MG recommended bioinformatic tools for NGS data analysis and provided bioinformatic support. KZ and MJ interpreted the data and wrote the manuscript. KD and ZD acquired funding, supervised the analyses and revised the manuscript.

Ethics approval and consent to participate

The authors followed all required ethical guidelines and received approval from the Ethics Committee of Jessenius Faculty of Medicine, Comenius University (IORG0004721) under project identification code EC 1525/2014. Written informed consent was obtained from all patients and the study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

The authors gained informed consent for publication of the dataset from participants at the point of recruitment to the

study and publication of these data does not compromise anonymity or confidentiality or breach local data protection laws, for the dataset to be considered for publication.

Competing interests

The authors declare that they have no competing interests.

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