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Use of Liquid Biopsy to Detect *PIK3CA* Mutation in Metastatic Breast Cancer

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Background: *PIK3CA* is associated with tumor progression, and the prevalence of *PIK3CA* mutation is high in breast cancer. Liquid biopsy offers convenient, noninvasive, and real-time insight into genetic alteration. In this study, we used liquid biopsy to detect *PIK3CA* mutations in patients with breast cancer.
Methods: We recruited patients with histologically confirmed breast cancer and distant metastases between April 2020 and September 2020. Circulating DNA was extracted from plasma (ctDNA) and exosomes (exoDNA). *PIK3CA* mutations (exons 9 and 20) were analyzed by droplet digital PCR.
Results: Of the 52 patients recruited, 16 had *PIK3CA* mutations in tumor tissue or blood: 9 had exon 9 mutations (E542K and E545K) and 8 had exon 20 mutations (H1047 L and H1047R). In 8 (15%) of the 52 patients, *PIK3CA* mutations were detected by liquid biopsies using ctDNA in 5 (9%), exoDNA in 6 (11%), and both ctDNA and exoDNA in 3 (6%). Of the 8 patients with *PIK3CA* mutations detected by liquid biopsies, 3 had no *PIK3CA* mutations in the primary tumors.
Conclusions: *PIK3CA* mutations can be detected by liquid biopsy even in patients with no *PIK3CA* mutations in their primary tumors; thus, combination analysis using tissue and liquid biopsies can provide clinically useful information for patients with breast cancer. (J Nippon Med Sch 2022; 89: 66–71)

Key words: breast cancer, liquid biopsy, genetic screening

Introduction

Breast cancer is classified in terms of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor type 2 (HER2) status, and Ki67, and comprises 4 immunohistochemical (IHC) tumor subtypes, namely, luminal A, luminal B, HER2-enriched type, and triple-negative (TN) type. Breast cancer is treated according to the subtype[1]; however, genetic alterations can substantially modify the effectiveness of treatment.

PIK3CA is associated with tumor progression and has a high mutation rate in breast (21-35%), colorectal (13-32%), and endometrial (24-32%) cancers[2–4]. There are 3 established mutation hot-spots, namely E545K, E542K, and H1047R/L, which are responsible for 70-80% of all *PIK3CA* mutations[5]. The prevalence of *PIK3CA* mutation is high in luminal and HER2-enriched breast cancer but low in TN breast cancer[2]. In addition, *PIK3CA* mutation is a cause of trastuzumab resistance[6]. In ER-positive patients, *PIK3CA* mutations are associated with favorable outcomes for patients treated with aromatase inhibitors[7]. PIK3CA mutations are targets and predictors of efficacy for new molecular targeted agents[8,9].

Heterogeneity, including spatial and temporal heterogeneity, makes cancer treatment challenging[10]. There is a high incidence of discrepancy in *PIK3CA* mutations between primary breast cancer tumors and metastases; 21/56 (37.5%) change their genotype from wild type to mutant and 11/44 (25.0%) lose mutations in metastases[11], highlighting the need to assess *PIK3CA* status in metas-

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Table 1 Patient characteristics

Age, median (range)	58 (34-79)
Estrogen receptor Positive:Negative	39:13
HER2 Positive:Negative	8:44
IHC subtype	
Luminal A:Luminal B (HER2-):Luminal B (HER2+):HER2-enriched:TN	25:11:3:5:8
Number of metastatic sites, median (range)	2 (1-4)
Resection of primary tumor	
Yes:No	33:19

Estrogen receptor–positive: cutoff was ≥1% positive tumor cells
HER2, human epidermal growth factor receptor 2; IHC, immunohistological

tatic lesions. However, traditional biopsies of metastatic liver and lung lesions involve numerous risks. In addition, adequate samples are not always available or accessible for tissue-based analysis. Even when available, tissue obtained during primary surgical resection or biopsy may not reflect current tumor molecular characteristics.

Liquid biopsy analyzes plasma-derived circulating tumor DNA (ctDNA) and offers less invasive, real-time insight into genetic alterations[12]. Exosomes are a distinct source of tumor DNA that may be complementary to conventional liquid biopsy DNA sources[13,14]. In this study, we aimed to detect *PIK3CA* mutations by means of liquid biopsy using ctDNA in patients with metastatic breast cancer. In addition, we attempted to detect *PIK3CA* mutations using exosome-derived DNA (exoDNA).

Materials and Methods

Patients

We prospectively recruited patients with histologically confirmed breast cancer with distant metastases between April and September 2020. Inclusion criteria for this study were age >20 years and an Eastern Cooperative Oncology Group performance status of 0 or 1. This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Review Committee of Nippon Medical School (Tokyo, Japan No. R1-07-1164). Written informed consent was obtained from all participants.

Extraction of Primary Breast Tumor Tissue DNA, CtDNA, and ExoDNA

DNA was purified from formalin-fixed, paraffin-embedded specimens of primary breast tumor by a QIAamp DNA Mini Kit (Qiagen, Limburg, the Netherlands) according to the manufacturer's recommendations.

Peripheral blood samples were transferred to BD Vacutainer EDTA tubes (Becton Dickinson, Oxford, UK) and processed within 2 h. Plasma was separated by centrifuging the blood at 3,000 × *g* for 10 min at 4°C, before being stored at −80°C until DNA extraction. ctDNA was extracted from 1 mL of plasma by using a Maxwell® RSC cfDNA Plasma Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. ctDNA concentrations were measured with a Qubit quantification assay (Thermo Fisher Scientific, Waltham City, MA, USA).

Exosomes were isolated from 800 µL of plasma using a Total Exosome Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol and resuspended in 300 µL of PBS. ExoDNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Limburg, the Netherlands) according to the manufacturer's protocol and eluted in 50 µL of elution buffer. ExoDNA concentrations were measured with a Qubit quantification assay (Thermo Fisher Scientific, Waltham City, MA, USA).

Droplet Digital PCR (ddPCR) Assay for *PICK3CA* Mutations

PIK3CA mutations (E542K, E545K, H1047 L, and H1047 R) were analyzed with the QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Read analysis was performed on a QX200TM Droplet Reader (Bio-Rad Laboratories), and data were analyzed using QuantaSoft 1.7 and QuantaSoft™ Analysis Pro 1.0 software (Bio-Rad Laboratories). The 5 positive droplets were used as a cutoff.

Results

Patient Characteristics

The clinicopathological features of the tumors sampled from selected patients with breast cancer are shown in **Table 1**. Fifty-two patients were enrolled in this study, and immunohistochemistry was used to determine IHC tumor subtype, as follows: luminal A (25 cases), luminal B (14 cases), HER2-enriched (5 cases), and TN (8 cases). Each subtype was defined is as follows: luminal A indicates ER-positive (cutoff, ≥1% positive tumor cells), PgR-

Table 2 Patients with *PIK3CA* mutation

Pt	Age	Subtype	Metastatic sites	tissueDNA	ctDNA	exoDNA	Interval[a)] (months)	Number of regimens	ET
1	70	Luminal A	Lung, Liver, LN	-	-	E542K	288	7	Yes
2	53	Luminal A	Liver, LN	-	H1047R	H1047R	109	5	Yes
3	57	Luminal A	Lung, Liver, Bone, Pleura	E542K	E542K	-	137	2	Yes
4	64	Luminal A	Bone, adrenal gland	E542K	E542K	-	2	2	Yes
5	34	Luminal A	Lung, LN	E545K	-	-	39	3	Yes
6	67	Luminal A	Lung, Liver, Bone, Brain	H1047R	-	-	0	2	No
7	57	Luminal A	Lung, Bone	E542K	-	-	1	1	No
8	69	Luminal B HER2-	Lung LN	-	-	H1047R	9	1	Yes
9	77	Luminal B HER2-	LN	E545K	-	-	60	6	Yes
10	75	Luminal B HER2-	LN	H1047R	-	-	33	10	Yes
11	57	Luminal B HER2-	Lung	H1047L	H1047L	H1047L	20	6	Yes
12	66	Luminal B HER2+	Skin	E542K H1047R	-	-	32	2	Yes
13	57	HER2-enriched	Pleura	E545K	-	-	69	2	No
14	65	HER2-enriched	Liver, Brain, Bone	E542K	-	E542K	14	2	No
15	57	TN	Liver, LN	H1047L	-	-	11	2	No
16	70	TN	Lung, Liver, LN, Bone	H1047R	H1047R	H1047R	0	1	No

ctDNA, circulating DNA; ET, endocrine therapy; exoDNA, exosomal DNA; LN, lymph node; Pt, patient; TN, triple negative
[a)] Interval between tissue sampling and liquid biopsy

positive (cutoff, ≥1% positive tumor cells), HER2-negative, and Ki67 labeling index of ≤20%; luminal B (HER2−) indicates ER-positive, PgR-positive or negative, and Ki67 labeling index of >20%; luminal B (HER2+) indicates ER-positive, PgR-positive or negative, and HER2-positive; HER2-enriched indicates ER-negative, PgR-negative, and HER2-positive; TN indicates ER-negative, PgR-negative, and HER2-negative. Of the 52 patients, 33 underwent resection of the primary tumor resection, and tumor tissue was collected by core needle biopsy from the remaining 19 patients. All patients were undergoing chemotherapy when blood samples were obtained.

***PIK3CA* Mutation in the Primary Tumor**

In total, 13 of the 52 patients had *PIK3CA* mutations in their primary tumor: 8 had exon 9 mutations (E542K and E545K) and 6 had exon 20 mutations (H1047 L and H 1047R) (**Table 2**). Only 1 patient had 2 mutations in *PIK3 CA* (E542K and H1047R). Of these 13 patients with *PIK3 CA* mutations in primary tumors, 10 (77%) were ER-positive; 5 were classified as luminal A and 5 were classified as luminal B. Variant allele frequency varied from 8.7% to 68.0%.

***PIK3CA* Mutation in Blood**

The interval between tissue sampling and liquid biopsy was 0-288 months (median 33 months). The median concentration of DNA was significantly higher in the plasma (236.5 ng/mL, range: 68.0-2,120) than that in the exosome (32.0 ng/mL, range 20.0-144.0) (**Fig. 1**). Among the 52 patients, *PIK3CA* mutations were detected with ctDNA in 5 patients (9%) (**Table 3A**), with exoDNA in 6 patients (11%), and with both ctDNA and exoDNA in 3 patients (6%) (**Table 2, 3B**). *PIK3CA* mutations were detected by using either ctDNA or exoDNA in 8 patients (15%) (**Table 2, 3C**). The sensitivity for detecting *PIK3CA* mutations was 31% (4/13) with ctDNA, 23% (3/13) with exoDNA, and 38% (5/13) with either ctDNA or exoDNA (**Table 3A, B, C**).

Discordance of Tumor Tissue and Liquid Biopsies

In the 8 patients with *PIK3CA* mutations detected by liquid biopsy, 5 had *PIK3CA* mutations in tumors. The interval between tissue sampling and liquid biopsy was 0, 2, 14, 20, and 137 months in these 5 patients. Of these

5 patients, 4 received 2 or fewer regimens of chemotherapy. The interval for the remaining 3 patients with no *PIK3CA* mutations in their tumors was 9, 109, and 288 months. Conversely, 2 of the 3 patients with no *PIK3CA* mutation in their tumor received 5 or 7 regimens of chemotherapy, and only 1 patient received 1 regimen of chemotherapy. Endocrine therapy was administered to 3 of 5 patients with *PIK3CA* mutation in their tumors and to 3 of 3 patients without the mutations in their primary tumors.

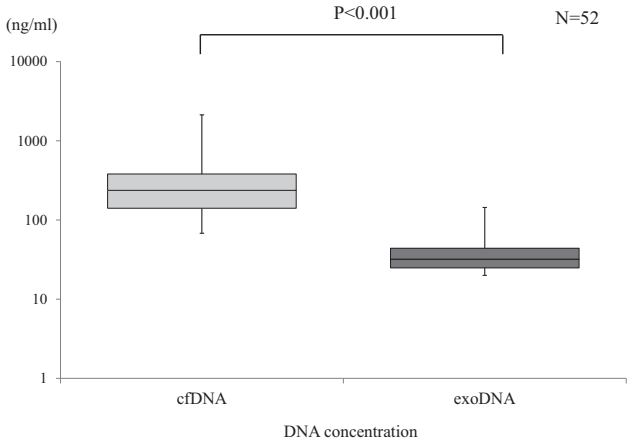

Fig. 1 The concentration of cfDNA was significantly higher than that of exosomal DNA.

In summary, of the 52 patients, *PIK3CA* mutations were detected in tumor or blood samples in 5/25 (20%) luminal A tumors, 5/14 (36%) luminal B tumors, 1/5 (20%) HER2-enriched tumors, and 2/8 (25%) TN tumors.

Discussion

This study identified 3 valuable and novel findings. First, although *PIK3CA* mutation can be detected by liquid biopsy, sensitivity is low. Second, heterogeneity relevant to *PIK3CA* mutation can be detected by liquid biopsy. Third, exoDNA is a promising source of information obtained by liquid biopsy. Similar to a previous report[2], the present study noted a *PIK3CA* prevalence of 25% (13/52). However, more patients can have *PIK3CA* mutations in their metastatic tumors, which are the main target of chemotherapy.

PIK3CA mutation can be detected by liquid biopsy;

Table 3A Sensitivity using ctDNA

	Tissue mutation (+)	Tissue mutation (–)
ctDNA Mutation (+)	4	1
ctDNA Mutation (–)	9	38
Sensitivity: 30.8%	Specificity: 97.4%	

ctDNA: circulating tumor DNA (circulating DNA extracted from plasma)

Table 3B Sensitivity using exoDNA

	Tissue mutation (+)	Tissue mutation (–)
exoDNA Mutation (+)	3	3
exoDNA Mutation (–)	10	36
Sensitivity: 23.1%	Specificity: 92.3%	

exoDNA: exosomal DNA (circulating DNA extracted from exosomes)

Table 3C Sensitivity using liquid biopsies (ctDNA and exoDNA)

	Tissue mutation (+)	Tissue mutation (–)
Liquid biopsies Mutation (+)	5	3
Liquid biopsies Mutation (–)	8	36
Sensitivity: 38.5%	Specificity: 92.3%	

Table 4 Reports of *PIK3CA* mutation detected by liquid biopsy

Author	Year	Number of patients	Number of patients with PIK3CA mutation in cancer tissue	Interval[a)]	Sensitivity (%)
Beaver JA[13)]	2014	29	14	Pretreatment	93
Rodriguez BJ[14)]	2019	29	23	Immediately	26
Nakauchi C[15)]	2016	17	4	50 months	75
Kodahl AR[16)]	2018	29	24	1.5 days	83
Tzanikou E[17)]	2019	16	9	Not available	44
The present study	2020	52	13	65 months	38

a) Interval between tissue sampling and liquid biopsy

however, sensitivity is low. The sensitivity for detection of *PIK3CA* mutation in the present study was 38%. We previously reported that *KRAS* mutations were detected in approximately 90% of patients who had metastatic colorectal cancer with *KRAS* mutations in their primary tumors[15]. Among patients with pancreatic cancer, the detection rate of *KRAS* mutations was 77.9%[16]. In terms of *PIK 3CA* mutations in patients with breast cancer, the detection rate for liquid biopsy was 26-93%[17–21] (**Table 4**). These studies included patients with varying background characteristics, which could explain the difference in sensitivity. Previous studies reported that prognosis was worse for ctDNA-positive patients and that ctDNA was not detected in patients with a low burden of metastatic disease[22–24]. Thus, potential false-negative results should be considered when using liquid biopsy to test for *PIK3CA* mutation.

Heterogeneity relevant to *PIK3CA* mutation can be detected by liquid biopsy. In the present study, 3/39 (8%) patients with *PIK3CA* mutations detected by liquid biopsies had no *PIK3CA* mutations in their primary tumors. A few studies reported heterogeneity of PIK3CA mutations between tumor tissue and liquid biopsy. Beaver et al. reported that none of the non-metastatic patients without a *PIK3CA* mutation had detectable *PIK3CA* mutations in ctDNA preoperatively[17]. Conversely, for metastatic disease, Nakauchi et al. showed that 2/13 (15%) patients had no *PIK3CA* mutation in their tumor[19]. Rodriguez et al. detected *PIK3CA* mutation in ctDNA from 1 (17%) of 6 patients without *PIK3CA* mutation in their tumor, and all 3 of these patients were ER positive, as was the case in our study[18]. All 3 of the present patients had received endocrine therapy, indicating that endocrine therapy can induce *PIK3CA* mutation. In addition, 2 of the 3 patients had been treated with a high number of chemotherapy regimens, suggesting that chemotherapy may also induce *PIK3CA* mutations. These results indicate heterogeneity in *PIK3CA* and that this heterogeneity can be detected by liquid biopsy.

Regarding the association between breast cancer subtype and *PIK3CA* mutations, previous reports[2] showed that the frequency of *PIK3CA* mutations is higher in luminal or HER2-enriched breast cancer; however, tin he present study, we found no significant difference in the frequency of *PIK3CA* mutations in relation to subtype. The effect of *PIK3CA* mutation on survival was reported to differ by subtype[7]. The present study did not investigate the association of treatment effect with *PIK3CA* mutation, which is a subject for future study.

In the present study, *PIK3CA* mutations were detected in 5 patients using ctDNA, 6 patients using exoDNA, and 8 patients using both. Allenson et al. reported that exoDNA outperformed ctDNA for detecting mutant *KRAS* in patients with pancreatic cancer[14]. However, to our knowledge, the present study is the first to use exoDNA to detect *PIK3CA* mutations. The results indicate that exosomes may be complementary to other DNA sources collected by liquid biopsy.

This study has several limitations. First, it included a limited number of patients at a single center, as in previous studies. Therefore, ctDNA and exoDNA results cannot be compared. Second, we did not evaluate the effect of detecting *PIK3CA* mutation using ctDNA or exoDNA on survival. Third, samples obtained before chemotherapy were not available; thus, it is unclear whether the heterogeneity detected in the present study is spatial or temporal.

In conclusion, although the sensitivity of liquid biopsy was limited, *PIK3CA* mutations were detectable. Liquid biopsy using exoDNA might improve the sensitivity of liquid biopsy. Moreover, *PIK3CA* mutations can be detected by liquid biopsy in patients with no *PIK3CA*.

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