Pathology & Oncology Research (2019) 25:1635–1643
https://doi.org/10.1007/s12253-019-00618-z

ORIGINAL ARTICLE

Drainage of Tumor-Derived DNA into Sentinel Lymph Nodes in Breast Cancer Patients

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Received: 29 October 2018 / Accepted: 15 February 2019 / Published online: 25 February 2019

Abstract

Circulating tumor DNA (ctDNA) is released from cancer cells by apoptosis or other mechanisms, and as tumor tissue contains both blood and lymphatic vessels, ctDNA can spread to local lymph nodes (LNs). We aimed to detect the tumor-derived free DNA in metastasis-free LNs in patients with breast cancers harboring the *PIK3CA*-H1047R mutation. One hundred twenty-three patients were evaluated and the *PIK3CA*-H1047R mutation was assayed in sentinel LNs (SLNs), non-SLNs without metastasis, and serum by digital PCR. The mutant DNA was more frequent in metastasis-free SLNs (21.6%) than in metastasis-free non-SLNs (8.6%; $P = 0.038$), and patients with mutation-positive SLNs were more likely to be positive for serum mutant DNA. Apoptosis in primary breast tumors was determined by TUNEL assay. The apoptotic index was significantly higher ($P = 0.003$) in patients with mutation-positive SLNs without metastasis (mean, 1.17%) than those with mutation-negative SLNs without metastasis (mean, 0.79%). It was also significantly higher ($P = 0.006$) in those with mutation-positive serum (mean, 1.41%) than in those with mutation-negative serum (mean, 0.86%). Furthermore, fragment size of *PIK3CA*-H1047R mutant DNA in metastatic-free SLN lysate used for the one-step nucleic acid amplification (OSNA) assay was short (<500 bp). These results support the theory that DNA is released from the primary tumor via apoptotic fragmentation. In conclusion, ctDNA is detectable in metastasis-free LNs and significantly more frequent in SLNs from patients with breast tumors harboring a high apoptotic index, consistent with drainage of ctDNA from apoptotic primary tumor cells into SLNs.

Keywords Circulating tumor DNA · Sentinel lymph node · *PIK3CA* mutation · Apoptosis

Introduction

Sentinel lymph node biopsy (SLNB) is used to determine axillary lymph node (LN) status in clinically node-negative breast cancer patients [1]. Sentinel LNs (SLNs) are the first regional LNs to which tumor cells metastasize through lymphatic vessels. Once established in SLNs, tumor cells can migrate downstream to non-SLNs in an orderly sequence [2–4]. The size of the SLN tumor burden is correlated with the extent of metastasis in non-SLNs [5, 6]. The percentage of false-negative SLNs, or metastasis to non-SLNs but not SLNs, is estimated at only 4.6% to 9.8% [7–9]. Circulating tumor DNA (ctDNA) is cell-free DNA in the peripheral blood that harbors tumor-specific genetic or epigenetic changes [10, 11]. It is present at low concentrations, but can be detected by BEAMing or digital PCR (dPCR) [12–14]. ctDNA is thought to be released from tumor cells into the vasculature by apoptosis, necrosis, or secretion but the mechanism has not been fully described [15–17]. Because tumor tissue contains both blood and lymphatic vessels, it is assumed that tumor-derived DNA can drain into SLNs. The study aim was to detect tumor-derived DNA in metastasis-free SLNs of breast cancer patients with tumors harboring the *PIK3CA*-H1047R mutation, which is a frequent mutation in primary breast cancers [14, 18–20]. The corresponding mutant ctDNA was assayed by dPCR in SLNs with the histologically confirmed absence of tumor cells. In addition, we evaluated the clinical and pathological features of breast cancer patients with tumor-derived DNA in metastasis-free SLNs.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-019-00618-z) contains supplementary material, which is available to authorized users.

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Materials and Methods

Patients and Samples

Patients with cT1-3 N0 breast cancers without systemic neoadjuvant therapy prior to SLNB and mastectomy or breast-conserving surgery at Osaka University Hospital between June 2000 and September 2011 were retrospectively included in this study. Patients who underwent SLNB and whose SLNs were examined by one-step nucleic acid amplification (OSNA) assay between November 2015 and April 2017 were also enrolled. Informed consent was obtained from each patient. The Ethical Review Board of Osaka University Hospital and the Osaka University Research Ethics Committee approved this study (approved date/number; 25May2016/#14,111 and 21Apr2017/#737).

SLNB was performed by a combination of dye (isosulfan blue, patent blue or indocyanine green) and radiocolloid (technetium-99 m tin colloid) or by dye alone. A representative frozen section from each SLN was prepared and evaluated intraoperatively for metastasis. If the section was positive, completion axillary lymph node dissection (cALND) was performed. The SLN tissue that remained after evaluation of the frozen section was formalin-fixed and paraffin-embedded (FFPE) and cut into 2 mm serial sections. The diagnosis was histologically confirmed in SLN sections stained with hematoxylin and eosin (HE) and immunohistochemically with anti-pan cytokeratin (CK) antibody [21]. One representative paraffin section was prepared from each non-SLN for histological evaluation [21]. In the OSNA assay, the SLN was homogenized in 4 ml of lysis buffer Lynorhag (Sysmex, Kobe, Japan), and 20 μl of lysate was used.

PIK3CA Mutation Assay in Primary Breast Tumors

Total DNA was extracted from frozen tumor tissue with DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) and, for tumor samples obtained between 2015 and 2017, DNA was extracted from FFPE tissue sections using QIAamp DNA FFPE kits (Qiagen), following the manufacturer's instructions. Real-time PCR assays of the *PIK3CA*-H1047R mutation were performed with a LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). The custom TaqMan primers and probes designed for the *PIK3CA* mutation are shown in Supplementary Table S1.

PIK3CA Mutation Analysis in LNs

The thickness of FFPE tissue sections that ensured not missing metastatic tumor cells was determined by measuring the diameter of 500 breast cancer cells in 10 metastatic SLNs (mean of 16 and range of 10–36 μm). As shown in Figs. 1, 4 μm sections for confirming absence of tumor cells by HE staining or CK immunohistochemistry and three 10 μm sections for DNA extraction were cut from each LN. Genomic DNA was extracted using QIAamp DNA FFPE kits (Qiagen) following the manufacturer's instructions and eluted into 30 μl. dPCR assays of the *PIK3CA* mutation were performed with a QuantStudio 3D dPCR system (Life Technologies, Carlsbad, CA) as previously described [14]. The primers and probes were same as those used in the real-time PCR assays (Supplementary Table S1). The copy number values were calculated by the QuantStudioTM 3D AnalysisSuite Software v1.1.3 (Life Technologies) using the call data of the chip.

PIK3CA Mutation Analysis of cfDNA in Serum

Blood samples were collected prior to surgery. Total DNA was extracted from serum using a QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's instructions. The *PIK3CA* mutation was assayed by dPCR as described above.

TUNEL Assay

Apoptosis in primary breast tumors was determined by TUNEL assay. It was performed with an in situ apoptosis detection kit (TaKaRa, Shiga, Japan) following the manufacturer's instructions with minor modifications. Serial 4 μm FFPE sections of breast tumor tissue were deparaffinized with xylene, rehydrated and treated with 15 μg/ml proteinase K (Roche Applied Science) for 15 min at room temperature. Endogenous peroxidase was inactivated with 3% H_2O_2 for 5 min before applying 50 μl of labeling reaction mixture consisting of TdT Enzyme and labeling-safe buffer for 60 min at 37 °C. TdT was absent from the reaction buffer in negative controls. Sections of rat mammary gland (#MK504, TaKaRa) were used as positive controls. For visualization, the sections were incubated with 70 μl of anti-fluorescein isothiocyanate (FITC) horseradish peroxidase (HRP)-conjugated antibody at 37 °C for 30 min, treated with diaminobenzidine (DAB), and counterstained with hematoxylin. Tumor cells with positive staining were counted in four high-power (×400) fields of each slide. The apoptotic index was calculated as the percentage of TUNEL-positive cells/number of total cancer cells.

Estimation of *PIK3CA*-Mutant DNA Fragment Size in SLNs

For estimating the fragment size of *PIK3CA*-mutant DNA in SLNs, SLN lysates prepared during the OSNA assay were used instead of DNA extracted from FFPE which is degraded by formalin fixation. The OSNA assay can detect the LN metastasis through amplification of *CK19* mRNA which is expressed in tumor cells but not in the normal cells of the LN, with the same accuracy as that of routine histological

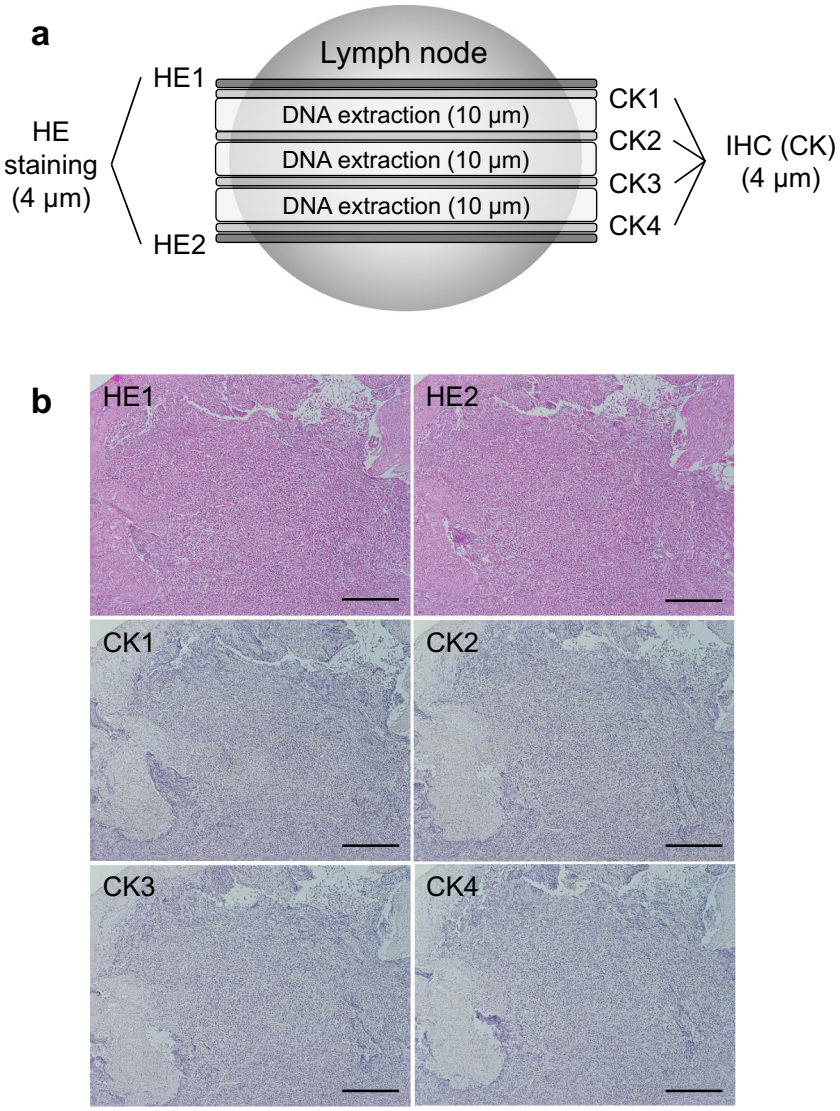

Fig. 1 Scheme of preparation of FFPE sections for histological examination and DNA extraction. **a:** Histological evaluation of paraffin blocks. DNA was extracted from three 10 μm lymph node sections, 4 μm serial sections adjacent to each 10 μm section were immunohistochemically stained for CK (CK1–4), and a series of 4 μm sections on the other side of the 10 μm section was stained with hematoxylin and eosin (HE1, 2) to confirm the absence or presence of tumor cells. **b:** Representative images of HE and IHC (CK) staining of a lymph node. Bars indicate 200 μm (×100)

examination [22]. Lysates from SLNs of patients with *PIK3CA* mutation-positive breast tumors were subjected to OSNA for detecting tumor cell metastasis and dPCR for detecting *PIK3CA* mutation. Total DNA was extracted from 100 μl of SLN lysates using a QIAamp Circulating Nucleic Acid Kit (Qiagen) and subjected to dPCR. We could detect an OSNA-negative (i.e., metastasis-free) and *PIK3CA* mutation-positive SLN which is supposed to have *PIK3CA*-mutant DNA derived from primary tumors but not from metastatic tumor cells in SLN, and it was used in the following study. In addition, OSNA-negative/*PIK3CA* mutation-negative SLN and OSNA-positive/*PIK3CA* mutation-positive SLN were used as a negative and positive control, respectively. The total DNA extracted from these SLNs was electrophoresed on a 2% agarose gel and separated into short (<500 bp) and long (>500 bp) fragments (Supplementary Fig. S1a). The DNA extracted from each fraction using MinElute Gel Extraction Kit (Qiagen) was subjected to dPCR.

Statistics

The statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Fisher's exact test was used to compare 2×2 and 2×3 groups. Differences in *PIK3CA*-mutant copy number in SLNs and non-SLNs were assessed with the Mann–Whitney U test or Wilcoxon signed rank test. The *t*-test was used to evaluate the association of the mutant DNA in SLN and apoptotic index. Differences were considered significant at $P < 0.05$.

Results

Screening for *PIK3CA* Mutation and Patient Selection

Of the 580 tumors from 580 breast cancer patients between 2000 and 2009 that were screened by real-time PCR, 123 (21.2%)

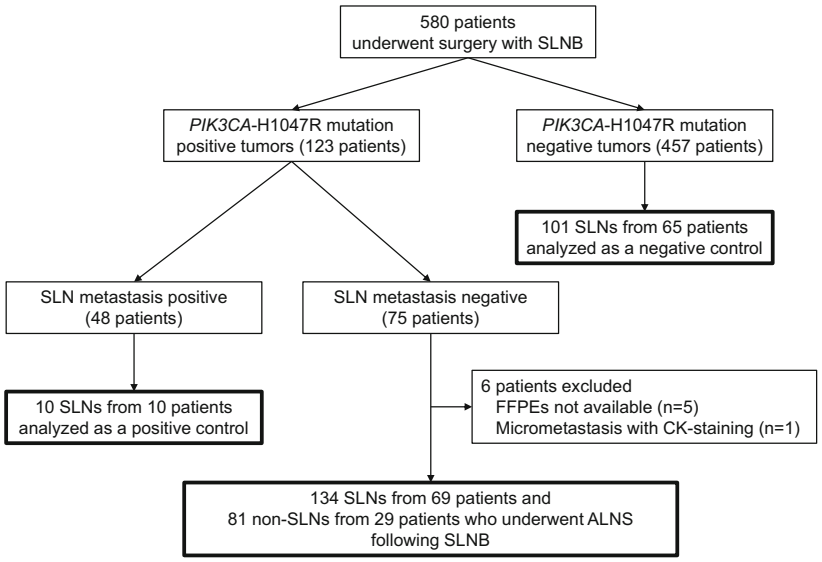

Fig. 2 Flowchart of patient disposition. SLNB, Sentinel lymph node biopsy. ALNS, Axillary lymph node surgery [completion axillary lymph node dissection (cALND) or axillary lymph node sampling]

were found to have the *PIK3CA* mutation. Of 123 patients, 75 (61%) did not have SLN metastases (Fig. 2). Six of the 75 patients were excluded and 134 SLNs from the remaining 69 patients were included in the analysis. Table 1 shows the clinicopathological characteristics of these 69 patients. The median follow-up after surgery was 13.4 years (range 6.4–18.2). Eighty-one non-SLNs were available from 29 patients with axillary LN surgery (ALNS), including cALND ($n = 7$) and

Table 1 *PIK3CA*-mutant DNA in SLN and clinical characteristics

		n	*PIK3CA*-mutant DNA in SLN		*P* value
			Positive	Negative	
Total patients		69	23	46	
Age	< 50	24	7	17	0.398
	≥ 50	45	16	29	
Tumor size	< 20 mm	42	14	28	0.601
	≥ 20 mm	27	9	18	
Histological subtype	Ductal	64	21	43	0.604
	Lobular	4	2	2	
	Others	1	0	1	
Histological grade	1, 2	58	20	38	0.465
	3	11	3	8	
LVI	Positive	17	5	12	0.468
	Negative	52	18	34	
ER / PgR	Positive	55	16	39	0.123
	Negative	14	7	7	
HER2	Positive	11	3	8	0.481
	Negative	47	16	31	
	Unknown	11	4	7	
PIK3CA-mutant DNA in serum	Positive	6	4	2	0.075
	Negative	57	16	41	
	Unknown	6	3	3	
Recurrence	Positive	7	3	4	0.429
	Negative	62	20	42	
BC related death	Positive	5	2	3	0.544
	Negative	64	21	43	

ER estrogen receptor, *PR* progesterone receptor
HER2 human epidermal growth factor receptor 2

sampling ($n = 22$). Ten SLNs with metastasis from ten patients with mutation-positive breast tumors were used as positive controls; 101 SLNs without metastasis from the 65 patients with *PIK3CA* mutation-negative breast tumors were used as negative controls.

Tumor-Derived DNA in SLNs and Non-SLNs

Fig. 3a shows the copy numbers of *PIK3CA*-mutant alleles detected in LNs by dPCR. Of 101 negative control SLNs, *PIK3CA*-mutant DNA was detectable in 14 SLNs (14%) with a very low copy number of mutant DNA (1.33–2.12 copies/assay), corresponding to one mutant call in the chip view. The samples were defined as positive for the mutation in the subsequent analysis, when >2.12 mutant copies/assay, i.e., >1 mutant calls per chip were detected. The *PIK3CA*-mutant DNA was detected in all of the ten positive controls (median of 710, and range of 70–4670 copies/assay). A total of 134 metastasis-free SLNs were obtained from 69 patients with *PIK3CA* mutation-positive breast tumors; the *PIK3CA*-mutant DNA was detected in 29 (21.6%), with a median of 4.6 and range of 2.7–39.5 copies/assay. A total of 81 metastasis-free non-SLNs were collected from 29 patients with *PIK3CA* mutation-positive breast tumors; the *PIK3CA*-mutant DNA was detected in 7 (8.6%), with a median of 3.1 and range of 2.9–5.8 copies/assay. The percentage of *PIK3CA* mutation-positive LN was significantly higher in SLNs (21.6%) than in non-SLNs (8.6%, $P = 0.038$), and the mutant copy number in mutation-positive LNs was significantly higher ($P = 0.010$) in SLNs than non-SLNs.

In the 29 patients with SLNB followed by ALNS and with SLNs and non-SLNs that were both negative for metastasis, *PIK3CA* mutation-positivity in non-SLNs was higher in patients who had mutation-positive SLNs (33%) than in those without them (6%); however, the difference was not statistically significant ($P = 0.078$, Table 2). The mean copy number of the *PIK3CA*-mutant DNA in the 13 patients with *PIK3CA* mutation-positive SLNs and/or non-SLNs was significantly higher in the SLNs (median of 2.2, and range of 0.0–23.8 copies/assay) than in the non-SLNs (median of 0.0 and range of 0.0–3.1, $P = 0.007$; Fig. 3b).

Clinicopathological Characteristics of Breast Cancer Patients with *PIK3CA* Mutation-Positive SLNs without Metastasis

PIK3CA mutation-positivity in SLNs was not associated with age, tumor size, histological subtype, histological grade, lymphovascular invasion, estrogen/progesterone receptor or human epidermal growth factor receptor 2 (HER2) status, or prognosis (Table 1). There was a trend for patients with *PIK3CA* mutation-positive SLNs to be positive for *PIK3CA* mutation in the serum as well ($P = 0.075$).

Correlation of Apoptosis in Primary Tumors with *PIK3CA*-Mutant DNA in both SLNs and Serum

Apoptosis in primary breast tumors with *PIK3CA*-mutatant DNA was assayed by TUNEL in the 60 patients with metastasis-free SLNs (Fig. 4a). The apoptotic index was

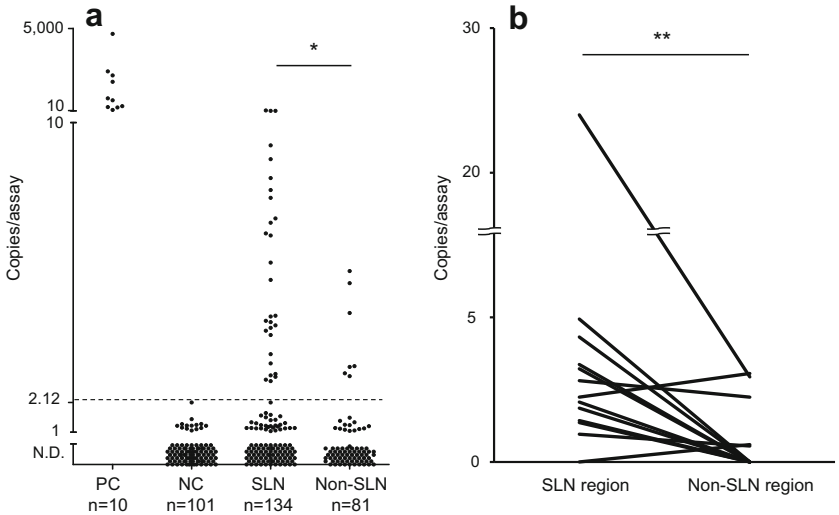

Fig. 3 *PIK3CA*-mutant DNA copies/assay in LNs. **a:** Copy numbers of *PIK3CA*-H1047R mutant alleles detected in LNs and assayed by dPCR are shown. Data are from 10 metastatic SLNs from patients with *PIK3CA* mutation in primary tumor as positive controls (PC), 101 SLNs from 65 patients without *PIK3CA* mutation in primary tumors as negative controls (NC), 134 SLNs without metastasis from 69 patients with *PIK3CA* mutation in primary tumors (SLN), and 81 non-SLNs without metastasis from 29 patients with ALNS (non-SLN). The cutoff for negative expression was 2.12 copies/assay (dotted line). ND, not detected. **b:** Comparison of *PIK3CA*-mutant DNA copy numbers in SLNs and non-SLNs. The mean copy numbers of *PIK3CA*-mutant DNA in SLNs and non-SLNs were compared in 13 patients with ALNS and harboring *PIK3CA*-mutant DNA in any LNs. *$P < 0.05$ by Mann–Whitney U test, **$P < 0.05$ by Wilcoxon signed rank test. SLN, sentinel lymph node

Table 2 Comparison of *PIK3CA*-mutant DNA in SLN and non-SLN (Patient based analysis; $n = 29$)

		PIK3CA-mutant DNA in SLN		
		Positive	Negative	Total
PIK3CA-mutant DNA in non-SLN	Positive	4 (33%)	1 (6%)	5
	Negative	8 (67%)	16 (94%)	24
	Total	12 (100%)	17 (100%)	29

significantly higher ($P = 0.003$) in the 17 patients with *PIK3CA* mutation-positive SLNs (mean, 1.17%) than in the 43 with *PIK3CA* mutation-negative SLNs (mean, 0.79%; Fig. 4b). The apoptotic index was also significantly higher ($P = 0.006$) in the 6 patients with *PIK3CA* mutation-positive serum (mean, 1.41%) than in the 49 with *PIK3CA* mutation-negative serum (mean, 0.86%; Supplementary Fig. S2).

Fragment Size of *PIK3CA*-Mutant DNA in LNs

Because circulating DNA is reported to be short (<180 bp) [16, 23], the fragment size of *PIK3CA*-mutant DNA in SLNs was assessed using SLN lysates used for OSNA assay. Total DNA were separated into short (<500 bp) and long (>500 bp) DNA fractions by agarose gel electrophoresis, and DNA from each fraction was assayed by dPCR (Supplementary Fig. S1a). *PIK3CA*-mutant DNA was undetectable in both short and long DNA fractions from negative control SLN and was detectable in both short and long DNA fractions (MAF = 0.20% and 0.23%, respectively) from positive control SLN (Fig. 5 and Supplementary Fig. S1b). Interestingly, *PIK3CA*-mutant DNA was detectable (MAF = 0.20%) in the short DNA fraction but not in the long DNA fraction from OSNA-negative and *PIK3CA* mutation-positive SLN. These results indicate that the fragment size of *PIK3CA*-mutant DNA in a metastasis-free SLN is short, although a metastasis-positive SLN contains long *PIK3CA*-mutant DNA fragments derived from metastatic tumor cells in SLNs.

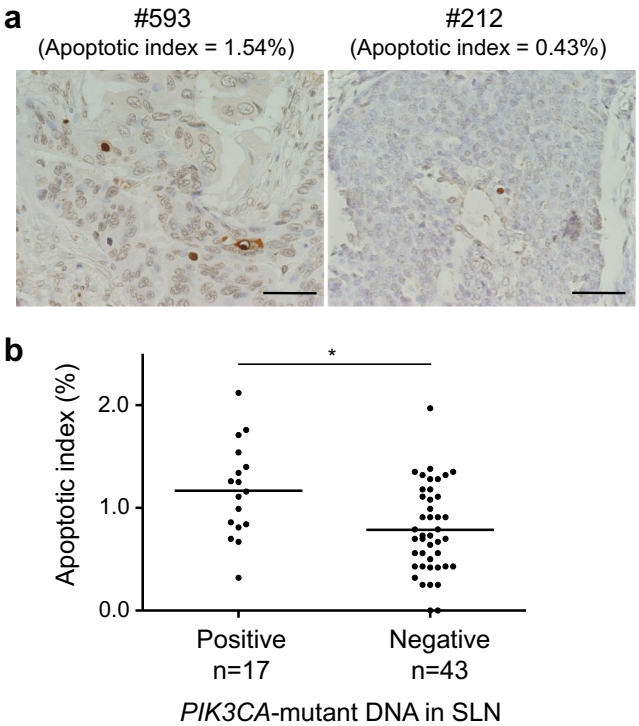

Fig. 4 **a:** Representative results of TUNEL staining in primary tumors. Apoptotic cells show positive TUNEL nuclei, which appear dark brown. A case (#593) with *PIK3CA*-mutant DNA (10.40 copies/assay) in SLN and a case (#212) without *PIK3CA*-mutant DNA in SLN. Bars indicate 50 μm (×400). **b:** *PIK3CA*-mutant DNA in SLN and apoptotic index in primary tumors. Apoptotic index (%) was assessed by TUNEL assay and compared in patients with SLNs containing *PIK3CA*-mutant DNA ($n = 17$) and in those with SLNs not containing *PIK3CA*-mutant DNA ($n = 43$). $^*P < 0.05$ by *t*-test. Bars indicate mean values

Discussion

We assayed the *PIK3CA* mutations in metastasis-free LNs from patients with *PIK3CA* mutation-positive breast tumors by dPCR. *PIK3CA*-mutant DNA was detected in 29 (22%) of 134 SLNs. Metastasis was confirmed or ruled out by histological evaluation of the 10 μm FFPE sections used for DNA extraction. To confirm the absence of tumor cells, both sides of each section were evaluated carefully by immunohistochemical staining (Fig. 1). It was unlikely that tumor cells were included in the section used for DNA extraction, indicating that the *PIK3CA*-mutant DNA detected in SLNs did not originate from tumor cells in SLNs. The results are consistent with drainage of tumor-derived (*PIK3CA*-mutant) DNA released from primary tumor tissues through lymphatic vessels into SLNs.

Non-SLNs were more frequently positive for tumor-derived DNA in patients with than in those without tumor-derived DNA-positive SLNs (33% versus 6%, Table 2), and the average copy

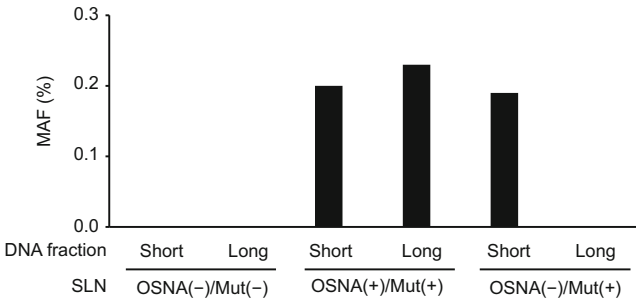

Fig. 5 Analysis of the fragment size of *PIK3CA*-mutant DNA in SLNs. *PIK3CA*-mutant allele frequency (MAF) in a short (<500 bp) or long (>500 bp) DNA fractions of OSNA lysates from OSNA-negative and *PIK3CA* mutation-negative (OSNA(−) / Mut(−)), OSNA(+) / Mut(+), and OSNA(−) / Mut(+) SLNs. DNA from SLNs was separated into short or long fractions by agarose gel electrophoresis, and DNA in each fraction was subjected to dPCR for the detection of *PIK3CA*-mutant DNA. MAF, mutant allele frequency; S, short fraction; L, long fraction

number was significantly higher in SLNs than in non-SLNs (Fig. 3b, $P = 0.007$). The anatomical difference in tumor-derived DNA copy number may support the hypothesis that DNA released from primary tumors can enter lymphatic vessels and drain into SLNs. We found one case in which tumor-derived DNA was not detected in SLNs but was positive in non-SLNs, which is in line with clinical studies that reported metastasis in non-SLNs but not in SLNs [7–9]. It is not impossible but very unlikely that tumor-derived DNA drains into axillary LNs through a different pathway than that taken by dye and/or radiolabel injected periareolarly. It is possible that tumor-derived DNA was not detected by dPCR because only a small portion of the SLN (three 10 μm sections) was used in the assay. This possibility is supported by histological evaluations that found metastasis-positive non-SLNs in patients with metastasis-negative SLNs [24].

ctDNA is reported to include about <180 bp, which corresponds to DNA resulting from apoptotic fragmentation [16, 25, 26]. Apoptosis is thus thought of as the most likely source of tumor-derived DNA in body fluids [17, 27], and is the rationale for determining whether the apoptotic index of the primary tumors was correlated with the presence of tumor-derived DNA in SLNs. The apoptotic index was significantly increased in patients with tumor-derived DNA-positive SLNs (Fig. 4b) and was also significantly associated with serum ctDNA (Supplementary Fig. S2). Serum ctDNA was more likely to be positive in patients with than without tumor-derived DNA in SLNs (Table 1, $P = 0.075$). These results support apoptosis as a source of tumor-derived DNA in SLNs and serum. In addition, our study on the fragment size of *PIK3CA*-mutant DNA demonstrated that *PIK3CA*-mutant DNA in a metastasis-free SLN was short (<500 bp). This observation seems to support the theory that DNA is released from the primary tumor via apoptotic fragmentation and migrates through the lymphatic vessels into the SLNs. A recent molecular study has suggested the association between the presence of tumor cells in SLNs and the detection of tumor-specific DNA methylation in OSNA lysates [28]. According to the present findings, however, it should be noted that the detected DNA may include not only DNA from metastatic tumor cells in SLNs but also ctDNA from the primary tumor, particularly when short amplicon PCR is used.

A potential study limitation is, firstly, the inclusion of tumor cells smaller than 10 μm in the FFPE sections assayed by dPCR that could have contributed to the *PIK3CA*-mutant DNA detected in LN tissue. However, we believe that to be very unlikely because the mean tumor cell size ($n = 500$) metastasizing to LNs was 10 μm or greater (mean, 16 μm, data not shown). It is reasonably suggested that *PIK3CA*-mutant DNA detected in metastasis-free LNs is derived from ctDNA present in the interstitial space or phagocytosed by normal LN cells, but not from the metastatic tumor cells in the LNs. Secondly, because the *PIK3CA* mutational status of primary tumors was screened using only a part of tumor tissues, the possibility cannot be ruled out that intratumoral genomic heterogeneity caused a mutational mismatch between primary tumors and LN metastases. However, we think such a possibility is very low since *PIK3CA* mutation is reported to occur early in tumor evolution [29, 30] so that the mutational mismatch between primary tumors and LN metastases is unlikely to happen.

In conclusion, tumor-derived DNA was detected in metastasis-free SLNs in breast cancer patients. The amount of tumor-derived DNA was significantly higher in SLNs than in non-SLNs. The presence of tumor-derived DNA was positively correlated with apoptosis in primary tumors and the DNA comprised short fragments, suggesting that tumor-derived DNA released from apoptotic tumor cells drained into SLNs via lymphatic vessels. The clinical impact of tumor-derived DNA in SLNs remains to be studied.

Acknowledgements This study was supported in part by the research funding from Novartis.

Funding This study was supported in part by the research funding from Novartis.

Compliance with Ethical Standards This study was approved by the Ethical Review Board of Osaka University Hospital and the Osaka University Research Ethics Committee (approved date/number; 25May2016/#14111 and 21Apr2017/#737).

Conflict of Interest Shinzaburo Noguchi has received research grant for other study from AstraZeneca and honoraria from AstraZeneca, Novartis, Pfizer, and Sysmex, and has been an advisor for AstraZeneca and Novartis. Naofumi Kagara received honoraria from AstraZeneca and Novartis. Yasuto Naoi received honoraria and Sysmex and research grant for other study from AstraZeneca and Sysmex. Masafumi Shimoda received honoraria from Novartis, Chugai and Eisai. Kenzo Shimazu received honoraria from AstraZeneca, Novartis, Sysmex and Kyowa Hakko Kirin. Seung Jin Kim received honoraria from AstraZeneca, Novartis, Chugai, Eisai, Kyowa Hakko Kirin, Pfizer, Takeda, Yakult, Taiho and Shimadzu Corporation. The other authors declare that they do not have a financial relationship with the organizations that sponsored the research.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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