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CCND1 Copy Number Variation in Circulating Tumor DNA from Luminal B Breast Cancer Patients

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Abstract. *Background/Aim: Abnormalities in the cyclin D1–CDK4/6 complex have been implicated in breast cancer proliferation and resistance to treatment. Recently, new drugs have been developed to target CDK4/6. Meanwhile, liquid biopsy has received great interest in oncology. In this study, we analyzed cyclin D1 gene (CCND1) copy number variation (CNV) in circulating tumor DNA (ctDNA) from luminal B breast cancer patients. Patients and Methods: This study included 31 patients with luminal B breast cancer who underwent resection. We analyzed CCND1 CNV in ctDNA by digital droplet PCR. Results: Of the 31 luminal B breast cancers, CCND1 CNV was positive in 5 cases. Patients with CCND1 CNV positivity had significantly shorter recurrence-free survival than patients with negative CCND1 CNV. Conclusion: CCND1 CNV in ctDNA was associated with poor prognosis in patients with luminal B breast cancer. This biomarker could be a useful prognostic factor.*

Breast cancer is broadly classified into subtypes according to the presence or absence of hormone receptors and HER2. Hormone receptor-positive breast cancers are further categorized into luminal A (low Ki67) and luminal B (high Ki67) according to their proliferative potential. Luminal B breast cancer has a poor prognosis without adjuvant therapy (1). Abnormalities in the cyclin D1–CDK4/6 complex, which regulates the G1 to S phase of the cell cycle, have been implicated in the growth of breast cancer and its resistance to treatment. In particular, luminal B breast cancer has been reported to have amplified cyclin D1 gene (*CCND1*) compared with other subtypes (2), but a means to easily assess cyclin D1 expression in a clinical setting has not yet been established. The U.S. Food and Drug Administration officially approved palbociclib CDK4/6 inhibitor in 2017 for hormone receptor-positive and HER2-negative unresectable and recurrent breast cancer, and in combination with aromatase inhibitor (letrozole), it was shown to significantly improved progression-free survival (PFS) and prolong overall survival (OS) (3, 4). CDK4/6 inhibitors represent an option for patients with unresectable or recurrent luminal B breast cancer who have a poor prognosis, and their therapeutic efficacy is expected to improve in clinical practice. However, a marker targeting the cyclin D1–CDK4/6 complex for effective administration of the drug is yet to be identified.

Liquid biopsy is a peripheral blood-based molecular diagnosis approach that avoids the need for an invasive tumor biopsy, in which the genomic profiling provides information on driver mutations, therapeutic resistance, and treatment response, and has gained increasing attention in oncology (5). In clinical practice, predictive markers for therapeutic response have been established for many cancer types, and in the case of breast cancer, Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 15-3 are used. Several reports indicated that the concentration of tumor-derived circulating tumor DNA (ctDNA) is more reflective of disease status than these existing markers (6, 7).

CCND1 is known as one of the most frequently altered genes and is associated with hyperactive PI3K/mTOR

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Key Words: Copy number variant, cyclin D1, circulating tumor DNA, breast cancer.

Table I. *Analysis of CCND1 CNV in ctDNA of 31 patients in cohort 1 and one healthy volunteer.*

Sample	Copies/20 μl Well	Measurements			STD	Theoretical values (n=2)			Judgement
		CNV	CNV Max	CNV Min		CNV	CNV Max	CNV Min	
DP_9266_PC	4,898.83	1.85	1.93	1.76	0.088	2.00	2.12	1.88	–
DP_9266_001	10,754.23	2.12	2.20	2.04	0.080	2.00	2.08	1.92	–
DP_9266_002	17,246.60	1.96	2.03	1.89	0.071	2.00	2.07	1.93	–
DP_9266_003	13,311.32	2.08	2.15	2.00	0.073	2.00	2.07	1.93	–
DP_9266_004	4,847.20	2.21	2.34	2.09	0.123	2.00	2.12	1.88	–
DP_9266_005	52,335.43	2.13	2.19	2.06	0.062	2.00	2.04	1.96	+
DP_9266_006	2,811.21	2.24	2.41	2.08	0.163	2.00	2.16	1.84	–
DP_9266_007	16,211.05	2.07	2.14	2.00	0.068	2.00	2.07	1.93	–
DP_9266_008	1,495.09	1.91	2.08	1.74	0.171	2.00	2.22	1.78	–
DP_9266_009	1,325.44	2.33	2.56	2.10	0.228	2.00	2.24	1.76	–
DP_9266_010	8,825.88	1.87	1.96	1.78	0.088	2.00	2.09	1.91	–
DP_9266_011	6,210.95	2.08	2.18	1.98	0.100	2.00	2.11	1.89	–
DP_9266_012	19,625.38	2.32	2.39	2.25	0.072	2.00	2.06	1.94	+
DP_9266_013	3,792.76	2.06	2.17	1.94	0.112	2.00	2.14	1.86	–
DP_9266_014	13,012.01	2.14	2.22	2.07	0.074	2.00	2.08	1.92	–
DP_9266_015	4,566.87	2.29	2.41	2.17	0.117	2.00	2.13	1.87	+
DP_9266_016	3,996.89	2.11	2.22	1.99	0.116	2.00	2.14	1.86	–
DP_9266_017	3,197.14	2.21	2.35	2.08	0.135	2.00	2.15	1.85	–
DP_9266_018	192.96	2.06	3.06	1.06	0.997	2.00	2.61	1.39	–
DP_9266_019	1,098.80	2.16	2.44	1.88	0.277	2.00	2.26	1.74	–
DP_9266_020	10,522.56	2.34	2.45	2.23	0.108	2.00	2.08	1.92	+
DP_9266_021	1,374.76	2.29	2.52	2.06	0.230	2.00	2.23	1.77	–
DP_9266_022	1,438.16	2.25	2.49	2.01	0.237	2.00	2.23	1.77	–
DP_9266_023	6,421.60	2.15	2.26	2.04	0.109	2.00	2.11	1.89	–
DP_9266_024	2,045.56	2.28	2.46	2.09	0.187	2.00	2.19	1.81	–
DP_9266_026	3,890.36	2.08	2.21	1.94	0.137	2.00	2.14	1.86	–
DP_9266_027	3,336.82	2.19	2.35	2.03	0.162	2.00	2.15	1.85	–
DP_9266_029	1,701.41	2.36	2.57	2.14	0.217	2.00	2.21	1.79	–
DP_9266_030	2,793.95	2.63	2.82	2.44	0.191	2.00	2.16	1.84	+
DP_9266_031	5,021.58	2.13	2.24	2.02	0.107	2.00	2.12	1.88	–
DP_9266_032	4,352.73	2.19	2.31	2.07	0.119	2.00	2.13	1.87	–
DP_9266_033	10,706.77	2.00	2.07	1.92	0.073	2.00	2.08	1.92	–

pathway, which was shown to lead to hormonal therapy resistance in the BOLERO-2 next generation research subgroup and The Cancer Genome Atlas network (8). By confirming the relationship between *CCND1* in ctDNA and *CCND1* in tumor tissue, we aimed to validate whether *CCND1* in ctDNA can be used as a therapeutic or prognostic marker. Several studies focused on the mutation and concentration of *ESR1* and *PIK3CA* in breast cancer (9, 10), but there are no reports on *CCND1* copy number variation (CNV) in both tissue and ctDNA in primary breast cancer. In this study, we first evaluated the association between *CCND1* CNV in ctDNA and the prognosis of patients with luminal B breast cancer (Cohort 1). Next, we assessed whether *CCND1* CNV in ctDNA reflected the biological characteristics of the lesion (Cohort 2).

Patients and Methods

Patients. We first evaluated the relationship between the CNV of *CCND1* in ctDNA and prognosis (Cohort 1) in 31 patients with luminal B breast cancer who underwent resection at Kyushu University Hospital (Fukuoka, Japan) between September 2006 and March 2009.

Next, we assessed whether *CCND1* CNV in ctDNA from peripheral blood reflected the biological characteristics of the tumor tissues (Cohort 2) in 15 patients with luminal B breast cancer who underwent resection at Kyushu University Hospital between January and June 2017. Tumor subtypes were identified using immunohistochemical (IHC) staining on tissue acquired by surgical resection. Estrogen receptor (ER)-positive or progesterone receptor (PR)-positive tissues were defined as tumors with ≥1% of tumour cells staining positive for ER or PR, respectively, as previously reported (11). Tumor specimens were defined as HER2-positive when HER2 IHC staining was scored as 3+ according to

Table II. *Clinicopathological characteristics of patients in Cohort 1.*

	Number of patients	(%)
Age		
Years, range (median)	27-79 (55)	
Sex		
Female	30	97
Male	1	3
pStage		
I	11	35.5
II	18	58
III	2	6.5
IV	0	0
Nuclear grade		
1	16	51.6
2	5	16.1
3	9	29
Unknown	1	3.2
Lymphatic invasion		
Negative	21	67.7
Positive	10	32.3
Vascular invasion		
Negative	31	100
Positive	0	0
Recurrence (1st)		
Negative	27	87
Positive (local)	2	6.5
Positive (distant)	2	6.5
Death		
Yes	3	9.7
No	28	90.3

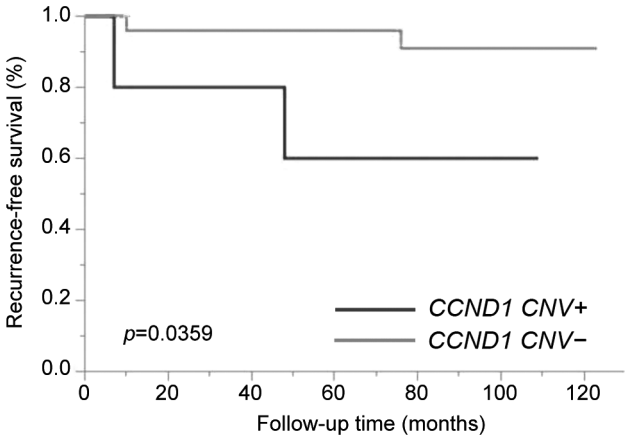

Figure 1. *Prognostic value of CCND1 CNV expression in ctDNA. Kaplan-Meier curves showing estimated RFS for CCND1 CNV expression in ctDNA. p-Value indicates the significance of the difference between the two groups.*

Table III. *Clinicopathological characteristics of 15 patients in Cohort 2.*

	Number of patients	(%)
Age		
Years, range (median)	38-84 (58.3)	
Sex		
Female	15	100
Male	0	0
pStage		
I	5	33.3
II	7	46.7
III	3	20
IV	0	0
Nuclear grade		
1	5	33.3
2	3	20
3	7	46.7
Unknown		3.2
Lymphatic invasion		
Negative	10	66.7
Positive	5	33.3
Vascular invasion		
Negative	14	93.3
Positive	1	6.7

standard criteria (12) or when *HER2* gene amplification was detected using fluorescence *in situ* hybridization. Cancer specimens were defined as luminal B when the Ki67 status was high (>20%) or the PR status was low (<20%) in ER-positive disease. The clinicopathological information of patients was collected retrospectively from the electronic medical records, including patient outcome and OS. The patients were treated according to the National Comprehensive Cancer Network Guidelines for the treatment of breast cancer (13), and the Clinical Practice Guidelines for Breast Cancer of the Japanese Breast Cancer Society (14).

The study conformed to the principles of the Declaration of Helsinki and was approved by the institutional review board of Kyushu University Hospital (no. 2020-591). Before surgery, patients provided comprehensive written consent, which stated that their medical information could be used for research purposes.

Samples. Patient blood samples (5 ml) were collected immediately before undergoing resection. In Cohort 1, blood samples were centrifuged at 3,000×*g* for 5 min to separate the serum, and the supernatant was collected and stored at −80˚C until further use. In Cohort 2, blood samples were centrifuged at 1,600×*g* for 20 min. The supernatant was then centrifuged at 12,000×*g* for 10 min to separate the plasma, and the supernatant was collected and stored at −80˚C until further use. In addition, we recruited one healthy volunteer who had no underlying disease and undertook venous puncture for collection of a blood sample. Formalin-fixed paraffin-embedded (FFPE) tumor tissues and frozen tissues were also collected after surgery.

DNA preparation from tissue samples and blood samples. DNA was extracted from each frozen tissue sample using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and DNA samples were confirmed to be >20 ng/μl in concentration. ctDNA was extracted using a QIAamp Circulating Nucleic Acid Kit (Qiagen) from plasma.

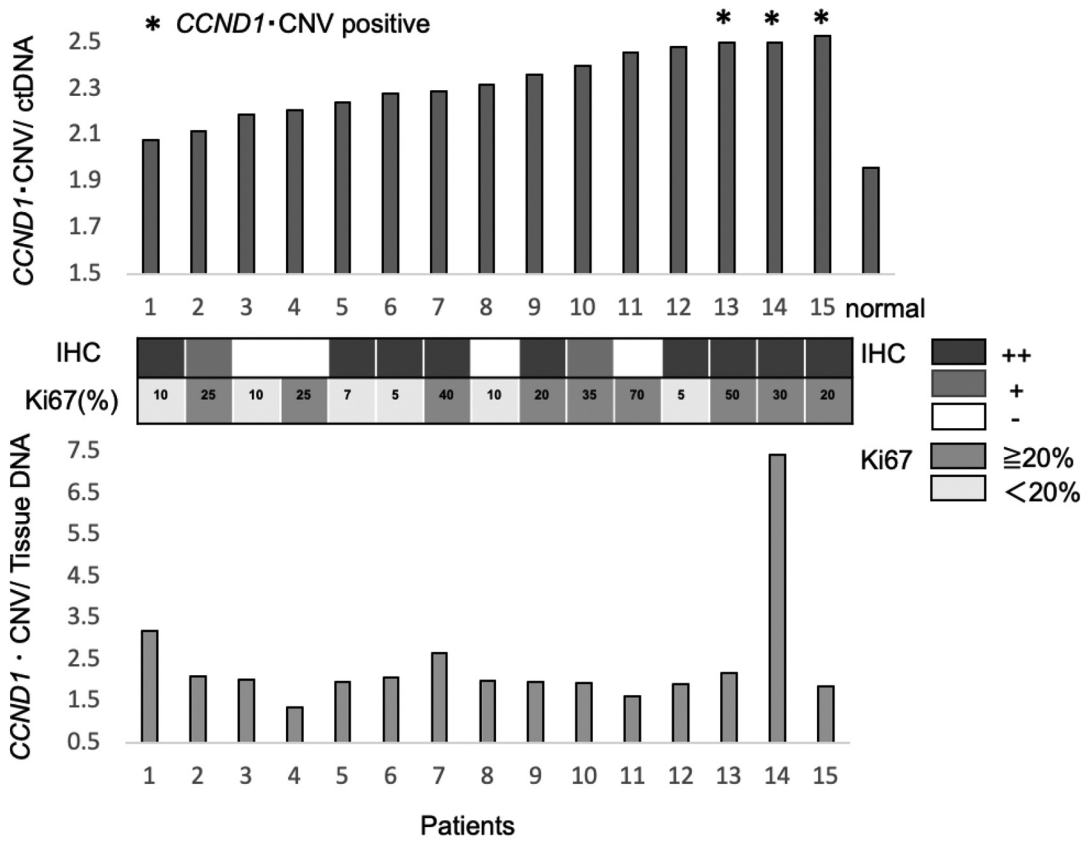

Figure 2. *Relationship between CCND1 CNV in plasma ctDNA, CCND1 CNV in tissue, and tissue IHC. Cases that were positive for CCND1 CNV in ctDNA (*) tended to be strongly positive for cyclin D1 protein and had high expression of Ki67.*

Digital droplet PCR for CCND1 copy number variation analysis. Digital droplet PCR (ddPCR) was performed to measure *CCND1* CNV in the DNA extracted from tissues and plasma using a QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocols. CN determination was performed as follows: From the results of ddPCR, the proximal curve to the input was drawn, and the theoretical value at CN=2 was calculated from the proximal curve. If the minimum value of the measured value was greater than the maximum value of the theoretical value, it was judged to be positive (Table I).

IHC staining. Anti-cyclin D1 antibody (monoclonal rabbit, SP4; Nichirei Bioscience, Tokyo, Japan) was used according to the manufacturer's protocol. Cyclin D1 positivity was defined as cyclin D1 expression in ≥10% of tumour cells (15). Furthermore, ≥50% cyclin D1 expression was considered to be strongly positive.

Statistical analysis. Logistic regression was used to compare continuous variables and χ^2 tests were performed to compare categorical variables between *CCND1*-positive and -negative groups. The survival endpoint was recurrence-free survival (RFS), which was defined as the time from surgery to recurrence, including both local relapse and metastatic disease. A survival curve was generated using the Kaplan-Meier method and compared with the log-rank test. A *p*-value of <0.05 was considered statistically significant. Statistical analysis was performed using JMP 16 (SAS Institute, Cary, NC, USA).

Results

The clinicopathological characteristics of Cohort 1, including age at diagnosis, pathological stage, histological characteristics, and outcomes, are listed in Table II. The mean age of the patients was 55 years (range=27-79 years) and one man (3%) was included. Most patients had clinical stage I (35.5%) or II (58.0%) disease and all had no vascular invasion. Of 31 patients, 27 (87%) were recurrence-free and four (13%) had local or distant recurrence. Five (16%) patients were *CCND1* CNV-positive. Three (11%) of the 27 recurrence-free cases were CNV-positive. In addition, two (50%) of four recurrent cases showed CNV positivity. A prognostic analysis with the endpoint of RFS showed that RFS was significantly lower in *CCND1* CNV-positive cases compared with negative cases (*p*=0.0359) (Figure 1).

The clinicopathological characteristics of patients in Cohort 2, including age at diagnosis, pathological stage, and histological characteristics, are listed in Table III. The mean age

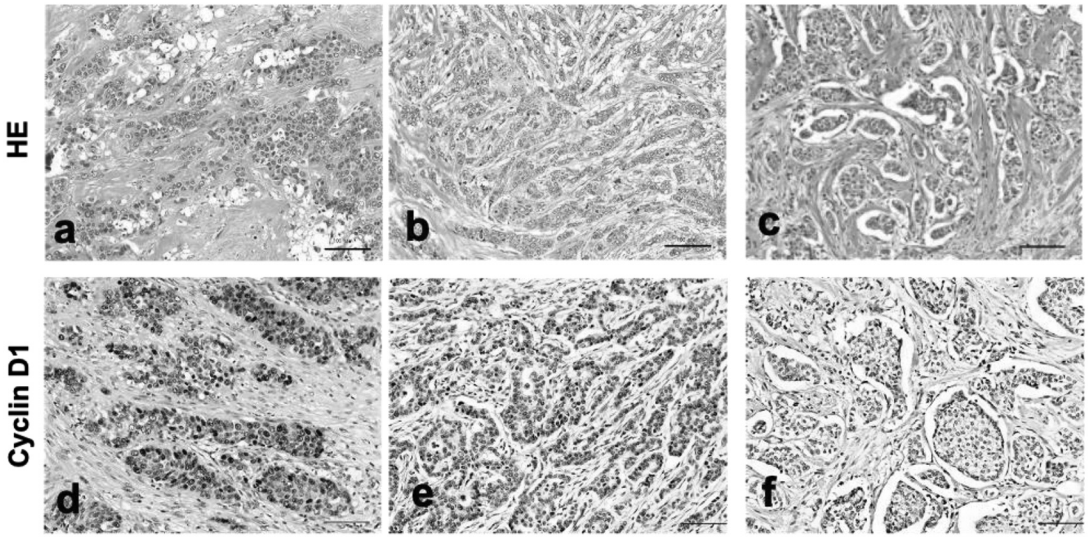

Figure 3. *Cyclin D1 expression in breast carcinomas analyzed by immunohistochemistry (magnification, ×200; bar, 100μm). (a–c) Haematoxylin-eosin staining shows tumors. (d) Cyclin D1 expression was strongly positive. (e) Cyclin D1 expression was moderately positive. (f) Cyclin D1 was not expressed.*

of the patients was 58.3 years (range=38-84 years) and all were female. Most patients had clinical stage I (33.3%) or II (46.7%) disease. The relationship between the CNV of *CCND1* in plasma ctDNA, CNV of *CCND1* in tissue, and tissue IHC are shown in Figure 2. There were three positive cases. Of the 15 luminal B breast cancer patients, 11 (73%) were cyclin D1-positive by IHC (Figure 3). Cases that were positive for *CCND1* CNV in ctDNA tended to be strongly positive for cyclin D1 protein by IHC and had high expression of Ki67. However, there was no match for *CCND1* CNV in ctDNA and tissue.

Discussion

To the best of our knowledge, this is the first report to show that it is possible to measure *CCND1* CNV in ctDNA and blood from patients with luminal B breast cancer. Analysis of *CCND1* CNV in ctDNA and the nature of the lesions was also performed but did not match the tissue *CCND1* CNV.

ctDNA in blood samples can offer essential advantages over tissue biopsies, including minimal invasiveness, access to tissue, use of preserved samples, and tumor heterogeneity, and as a result, is used to guide treatment decisions more quickly. ctDNA detection research is of great significance to the early diagnosis, prognostic evaluation, disease monitoring, and individualized treatment of breast cancer (7). Cyclin D1 over-expression occurs in half of all breast cancers (8, 16-18). In particular, it has been reported that *CCND1* is amplified in luminal B breast cancer (2). The prognostic utility of associated proteins has great potential, with over-expression associated with favorable prognosis

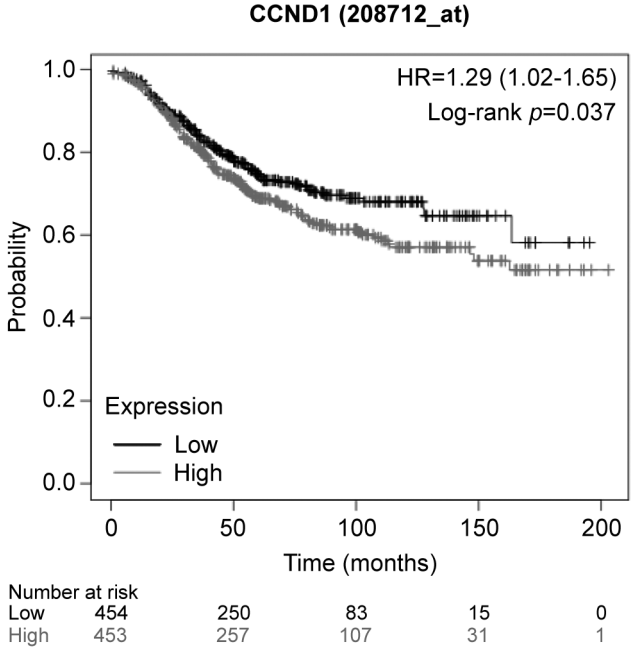

Figure 4. *Prognostic value of CCND1 mRNA expression in the Kaplan-Meier plotter (kmplot.com). Kaplan-Meier curve showing estimated RFS for CCND1 mRNA expression in luminal B breast cancer patients (n=904).*

(19, 20) and gene amplification signifying poor prognosis (21). This study showed that RFS was significantly lower in cases that were positive for *CCND1* CNV compared with those that were negative. The threshold for determining the

increase or decrease in copy number is difficult because it varies depending on various factors such as sample quality and quantity, and the combination of target and reference probes. In the present study, we generated an approximate curve from the ddPCR results and calculated theoretical values from the curve, and then used them as the basis for determining the increase or decrease in copy number.

When verified at the mRNA level using the Kaplan-Meier plotter, high *CCND1* mRNA expression was significantly correlated with shorter RFS among luminal B breast cancer patients (Figure 4). In the BOLERO-2 study, genetic alternations in the PI3K/mTOR pathway and cell-cycle control genes (*CCND1*, *CDK4*, *CDK6*, and *CDKN2A*) and amplification of *CCND1* had minimal benefit on the progression-free survival gain with mTOR inhibitor (everolimus) (8), which supported our study data.

The concordance trend of ctDNA and tissue *CCND1* CNV in this study is shown in Figure 2. However, in a study without breast cancer by Nakamura *et al*. (GOZILA study), ctDNA-based testing demonstrated 97%-100% concordance with tissue-based testing for identifying actionable mutations (22). In a study of breast cancer by Turner *et al*. (plasmaMATCH trial), there was 98%-100% concordance (23). Although the difference between primary and metastatic lesions may affect the result, further studies on ctDNA for primary breast cancer are needed.

This study has some limitations. First the sample size was small because we focused on a limited population. Second, it included only retrospectively collected cases. Increasingly, however, disease-tracking markers targeting the cyclin D1–CDK4/6 complex will be required for effective drug administration. This study suggests that the use of ctDNA may enable minimally invasive and real-time monitoring. In conclusion, we were able to detect *CCND1* CNV in ctDNA from patients with luminal B breast cancer. Positive *CCND1* CNV may be associated with worse survival.

Conflicts of Interest

There are no conflicts of interest regarding this study.

Authors' Contributions

AS and M Kubo wrote the manuscript; M Kubo, M Kai and K Kurata designed the study; AS, K Kurata, UT, SH, YH, HK, K Kaneshiro, and MY collected samples; AS and K Kurata performed the experiments and analysed data; MN provided intellectual input. All Authors reviewed the manuscript draft, revised it critically regarding the intellectual content, and approved the final version of the manuscript to be published.

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References

1 Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, Quackenbush JF, Stijleman IJ, Palazzo J, Marron JS, Nobel AB, Mardis E, Nielsen TO, Ellis MJ, Perou CM and Bernard PS: Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol *27(8)*: 1160-1167, 2009. PMID: 19204204. DOI: 10.1200/JCO.2008.18.1370

2 Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature *490(7418)*: 61-70, 2012. PMID: 23000897. DOI: 10.1038/nature11412

3 Finn RS, Martin M, Rugo HS, Jones S, Im SA, Gelmon K, Harbeck N, Lipatov ON, Walshe JM, Moulder S, Gauthier E, Lu DR, Randolph S, Diéras V and Slamon DJ: Palbociclib and letrozole in advanced breast cancer. N Engl J Med *375(20)*: 1925-1936, 2016. PMID: 27959613. DOI: 10.1056/NEJMoa1607303

4 Slamon DJ, Neven P, Chia S, Jerusalem G, De Laurentiis M, Im S, Petrakova K, Valeria Bianchi G, Martín M, Nusch A, Sonke GS, De la Cruz-Merino L, Beck JT, Ji Y, Wang C, Deore U, Chakravartty A, Zarate JP, Taran T and Fasching PA: Ribociclib plus fulvestrant for postmenopausal women with hormone receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer in the phase III randomized MONALEESA-3 trial: updated overall survival. Ann Oncol *32(8)*: 1015-1024, 2021. PMID: 34102253. DOI: 10.1016/j.annonc.2021.05.353

5 Nakamura M, Kageyama SI, Seki M, Suzuki A, Okumura M, Hojo H, Motegi A and Akimoto T: Liquid biopsy cell-free DNA biomarkers in patients with oligometastatic colorectal cancer treated by ablative radiotherapy. Anticancer Res *41(2)*: 829-834, 2021. PMID: 33517288. DOI: 10.21873/anticanres.14835

6 Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, Dunning MJ, Gale D, Forshew T, Mahler-Araujo B, Rajan S, Humphray S, Becq J, Halsall D, Wallis M, Bentley D, Caldas C and Rosenfeld N: Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med *368(13)*: 1199-1209, 2013. PMID: 23484797. DOI: 10.1056/NEJMoa1213261

7 Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, Parkinson C, Chin SF, Kingsbury Z, Wong AS, Marass F, Humphray S, Hadfield J, Bentley D, Chin TM, Brenton JD, Caldas C and Rosenfeld N: Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature *497(7447)*: 108-112, 2013. PMID: 23563269. DOI: 10.1038/nature12065

8 Hortobagyi GN, Chen D, Piccart M, Rugo HS, Burris HA 3rd, Pritchard KI, Campone M, Noguchi S, Perez AT, Deleu I, Shtivelband M, Masuda N, Dakhil S, Anderson I, Robinson DM, He W, Garg A, McDonald ER 3rd, Bitter H, Huang A, Taran T, Bachelot T, Lebrun F, Lebwohl D and Baselga J: Correlative analysis of genetic alterations and everolimus benefit in hormone receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer: Results from BOLERO-2. J Clin Oncol *34(5)*: 419-426, 2016. PMID: 26503204. DOI: 10.1200/JCO.2014.60.1971

9 Nicolini A, Ferrari P and Duffy MJ: Prognostic and predictive biomarkers in breast cancer: Past, present and future. Semin Cancer Biol *52(Pt 1)*: 56-73, 2018. PMID: 28882552. DOI: 10.1016/j.semcancer.2017.08.010

10 Sim SH, Yang HN, Jeon SY, Lee KS and Park IH: Mutation analysis using cell-free DNA for endocrine therapy in patients with HR+ metastatic breast cancer. Sci Rep *11(1)*: 5566, 2021. PMID: 33692409. DOI: 10.1038/s41598-021-84999-9

11 Kurata K, Kubo M, Kai M, Mori H, Kawaji H, Kaneshiro K, Yamada M, Nishimura R, Osako T, Arima N, Okido M, Oda Y and Nakamura M: Microsatellite instability in Japanese female patients with triple-negative breast cancer. Breast Cancer *27(3)*: 490-498, 2020. PMID: 31907878. DOI: 10.1007/s12282-019-01043-5

12 Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical Oncology. and College of American Pathologists: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol *31(31)*: 3997-4013, 2013. PMID: 24101045. DOI: 10.1200/JCO.2013.50.9984

13 NCCN Clinical Practice Guidelines of Breast Cancer, Version 5 (2020). Available at: https://www2.tri-kobe.org/nccn/guideline/breast/english/brest.pdf [Last accessed on March 31, 2022]

14 Clinical Practice Guideline of Breast Cancer by the Japanese Breast Cancer Society, Version 3 (2018). Available at: https://jbcs.xsrv.jp/guidline/2018/ [Last accessed on March 31, 2022]

15 Joe AK, Memeo L, Mckoy J, Mansukhani M, Liu H, Avila-Bront A, Romero J, Li H, Troxel A and Hibshoosh H: Cyclin D1 overexpression is associated with estrogen receptor expression in Caucasian but not African-American breast cancer. Anticancer Res *25(1A)*: 273-281, 2005. PMID: 15816548.

16 Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CK, Musgrove EA and Sutherland RL: Expression and amplification of cyclin genes in human breast cancer. Oncogene *8(8)*: 2127-2133, 1993. PMID: 8336939.

17 Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D and Peters G: Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res *54(7)*: 1812-1817, 1994. PMID: 8137296.

18 Ormandy CJ, Musgrove EA, Hui R, Daly RJ and Sutherland RL: Cyclin D1, EMS1 and 11q13 amplification in breast cancer. Breast Cancer Res Treat *78(3)*: 323-335, 2003. PMID: 12755491. DOI: 10.1023/a:1023033708204

19 Radenkovic S, Konjevic G, Nikitovic M, Stojanovic Rundic S, Plesinac Karapandzic V, Milovic Kovacevic M and Jurisic V: Evaluation of Cyclin D1 expression by western blotting methods and immunohistochemistry in breast cancer patients. J BUON *26(2)*: 475-482, 2021. PMID: 34076995.

20 Siraj AK, Parvathareddy SK, Annaiyappanaidu P, Ahmed SO, Siraj N, Tulbah A, Al-Dayel F, Ajarim D and Al-Kuraya KS: High expression of Cyclin D1 is an independent marker for favorable prognosis in Middle Eastern breast cancer. Onco Targets Ther *14*: 3309-3318, 2021. PMID: 34040395. DOI: 10.2147/OTT.S309091

21 Bièche I, Olivi M, Noguès C, Vidaud M and Lidereau R: Prognostic value of CCND1 gene status in sporadic breast tumours, as determined by real-time quantitative PCR assays. Br J Cancer *86(4)*: 580-586, 2002. PMID: 11870541. DOI: 10.1038/sj.bjc.6600109

22 Nakamura Y, Taniguchi H, Ikeda M, Bando H, Kato K, Morizane C, Esaki T, Komatsu Y, Kawamoto Y, Takahashi N, Ueno M, Kagawa Y, Nishina T, Kato T, Yamamoto Y, Furuse J, Denda T, Kawakami H, Oki E, Nakajima T, Nishida N, Yamaguchi K, Yasui H, Goto M, Matsuhashi N, Ohtsubo K, Yamazaki K, Tsuji A, Okamoto W, Tsuchihara K, Yamanaka T, Miki I, Sakamoto Y, Ichiki H, Hata M, Yamashita R, Ohtsu A, Odegaard JI and Yoshino T: Clinical utility of circulating tumor DNA sequencing in advanced gastrointestinal cancer: SCRUM-Japan GI-SCREEN and GOZILA studies. Nat Med *26(12)*: 1859-1864, 2020. PMID: 33020649. DOI: 10.1038/s41591-020-1063-5

23 Turner NC, Kingston B, Kilburn LS, Kernaghan S, Wardley AM, Macpherson IR, Baird RD, Roylance R, Stephens P, Oikonomidou O, Braybrooke JP, Tuthill M, Abraham J, Winter MC, Bye H, Hubank M, Gevensleben H, Cutts R, Snowdon C, Rea D, Cameron D, Shaaban A, Randle K, Martin S, Wilkinson K, Moretti L, Bliss JM and Ring A: Circulating tumour DNA analysis to direct therapy in advanced breast cancer (plasmaMATCH): a multicentre, multicohort, phase 2a, platform trial. Lancet Oncol *21(10)*: 1296-1308, 2020. PMID: 32919527. DOI: 10.1016/S1470-2045(20)30444-7

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