Molecular Barcode Sequencing of the Whole Ligand Binding Domain of the ESR1 Gene in Cell-Free DNA from Patients with Metastatic Breast Cancer

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ESR1 mutations in breast cancer are known as one of the mechanisms of resistance to aromatase inhibitors. These mutations often occur in the hotspot regions in the ligand binding domain (LBD), but comprehensive mutational analysis has shown that mutations are observed throughout the whole LBD. We previously developed a molecular barcode sequencing (MB-NGS) technique to detect ESR1 hotspot mutations in plasma with high sensitivity. In this study, we have developed a multiplex MB-NGS assay that covers the whole LBD of ESR1. The assay demonstrated that the background errors in the plasma DNA of 10 healthy controls were below 0.1%; thus, the limit of detection was set at 0.1%. We analyzed the plasma DNA of 54 patients with estrogen receptor–positive metastatic breast cancer. Seventeen mutations were detected in 13 patients (24%), with variant allele frequencies ranging from 0.13% to 10.67%, including six rare mutations with a variant allele frequency <1.0% and a novel nonhotspot mutation (A312V). Three patients had double mutations located in the same amplicons, and it was revealed that the double mutations were located in different alleles. ESR1 hotspot mutations were associated with a longer duration of aromatase inhibitor treatment under metastatic conditions and to liver metastasis. The multiplex MB-NGS assay is useful for the sensitive and comprehensive detection of mutations throughout the whole LBD of ESR1. Our assay can be applied to any specific target region of interest using tailor-made primers and can result in minimized sequencing volume and cost.

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Introduction

ESR1 mutations in breast cancer are known as one of the mechanisms of resistance to aromatase inhibitors (AIs). These mutations occur in approximately 30% of AI-treated metastatic breast cancers (MBCs) and accumulate in the hotspot regions at codons 536, 537, and 538 in the ligand binding domain (LBD) [1–3]. The majority of recent clinical studies have focused on these hotspot mutations, which are mostly assayed using cell-free DNA in plasma (liquid biopsy) by taking advantage of dPCR or BEAMing [4–7]. However, comprehensive mutational analysis of the complete ESR1 gene has revealed that almost all ESR1 mutations are seen in its LBD and that 76% of them are located in the hotspots [2,3,8–13], indicating that the analysis of only the hotspot mutations is not sufficient since 24% of ESR1 mutations might be overlooked. Thus, an assay for detecting the ESR1 mutations in the entire LBD with high sensitivity needs to be developed.

We have previously reported the use of conventional NGS for the detection of novel nonhotspot ESR1 mutations [14], but such a conventional NGS analysis is less sensitive than dPCR and thus is likely to miss a significant proportion of ESR1 mutations. Then, we introduced a molecular barcode technique [15,16] to achieve improved sensitivity and specificity (MB-NGS); with this technique, we could show that ESR1 mutations were detected with a high sensitivity (detection limit: 0.1%). However, only a single amplicon (114 bp) harboring the mutation hotspots at codons 536, 537, and 538 was analyzed in that study [17]. The analyzed region accounts for only 16% of the LBD, leaving the remaining 84% not screened. Therefore, in the present study, we attempted to develop multiplex MB-
NGS for a comprehensive screening of \( ESR1 \) mutations in the LBD with high sensitivity.

**Materials and Methods**

**Patients and Samples**

Plasma samples were collected from 54 patients with MBC who were treated at Osaka University Hospital between 2000 and 2018. Twenty of these 54 patients were the same as those analyzed in our previous study \[17\]. Clinicopathological characteristics of the patients are shown in Table 1. Forty-nine patients had recurrent MBC, and five patients had primary MBC. Positive ER status (Allred score ≥ 3 \[18\]) was confirmed in primary or recurrent tumors for all cases. The median disease-free interval of these 54 patients was 42.8 months (range: 1.9-223.5). Forty-seven patients were diagnosed with pri-

**DNA Extraction**

Plasma was separated from whole blood by centrifugation for 10 minutes at 3000 (1840 G) rpm and stored at −80°C until further use. The samples were centrifuged again for 10 minutes at 13,300 (16,000 G) rpm prior to DNA extraction to remove debris. Cell-free DNA was isolated from 2 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) or the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions, and eluted in 100 μl.

**Library Preparation for MB-NGS**

Assignment of MBs and adaptors (Rd1SP, Rd2SP, P5, and P7) was performed with PCR as previously reported \[17\], and the primers used for the library preparation are shown in Supplementary Table 1. The first PCR amplified the targeted region of \( ESR1 \)-LBD and assigned a 15-base barcode (BDHVBDHVBDHVBDH). Eleven sets of primers covering \( ESR1\)-LBD (c.928-c.1641) were designed with a median amplicon size of 135 bp (range: 130-145) using Primer3 [http://bioinfo.ut.ee/primer3/] or DesignStudio [https://designstudio.illumina.com/]. Primers were divided into two sets including amplicons 01/03/04/07/08/11 and 02/05/06/09/10. The first PCR was performed in a 20-μl reaction containing 10 μl of template DNA, 5× Phusion HF buffer (NEB, Ipswich, MA), 0.9 U of Phusion polymerase (NEB), 250 μM dNTPs, and each set of primers. Each primer concentration was optimized to reduce primer dimer formation and to equalize the molecular-barcode family numbers of each amplion (Supplementary Table 1). The cycling conditions were 1 cycle of 98°C for 30 seconds; 15 cycles of 98°C for 10 seconds, 60°C for 2 minutes, and 72°C for 30 seconds; and 1 cycle of 72°C for 10 minutes. The excess barcode primers were digested with 25 U of Exonuclease-I and 10× Exonuclease-I reaction buffer (NEB) in a 25-μl reaction at 37°C for 1 hour following heat inactivation at 98°C for 5 minutes. Adaptor primers with P5/P7 sequences (0.05 μM each) were added to the first PCR product, and the second PCR was performed in a 28-μl reaction volume. The second PCR was performed using forward and reverse primers including 2 and 24 different sample indi-

**Data Analysis**

The variant detection analysis was performed in a similar manner to that described in the previous study \[17\], with some modifications. Briefly, the quality of sequence reads was confirmed using FastQC, and low-quality bases (Q < 30) and assembled sequences with an unideal length were trimmed using PEAR 0.9.6. The reads whose MB sequences, the first 15 bp of assembled sequences, did not match with “BDHVBDHVBDHVBDH” were removed. Extracted assembled sequences were clustered into each family containing sequences with the same MB se-

### Table 1

|                        | Total          | Plasma ESR1 Mutation | \( P \)     |
|------------------------|----------------|----------------------|-------------|
| Number                 | 54             | 13                   | 41          |
| Age at blood sampling (years) | Mean (range)   | 56.5 (30-74)         | 56 (35-66)  | 57 (30-74)  | .348*       |
| Estrogen receptor      | Positive       | 54                   | 13          | 41          | 1.000*      |
|                        | Negative       | 0                    | 0           | 0           |            |
| Progesterone receptor  | Positive       | 40                   | 9           | 31          | .572*       |
|                        | Negative       | 11                   | 3           | 8           |            |
|                        | Unknown        | 3                    | 1           | 2           |            |
| HER2 status            | Positive       | 5                    | 0           | 5           | .321†       |
|                        | Negative       | 49                   | 13          | 36          |            |
| Primary or recurrent   | Primary        | 5                    | 1           | 4           |            |
|                        | Recurrent      | 49                   | 12          | 27          |            |
| DFI of recurrent breast cancer (months) | Median (range) | 42.8 (1.9-223.5)     | 52.3 (12.7-177.1) | 35.6 (1.9-223.5) | .140*       |
| AI treatment           | No AI          | 7                    | 1           | 6           | .045†       |
|                        | Adjuvant AI only | 11              | 0           | 11          |            |
| AI for MBC             | For MBC        | 36                   | 12          | 24          |            |
| Duration (month) of AI for MBC | Median (range) | 5.6 (0.0-74.8)      | 30.2 (0.0-74.8) | 2.9 (0.0-35.6) | <.001*       |

| **HER2**, human epidermal receptor 2; DFI, disease-free interval; AI, aromatase inhibitor; MBC, metastatic breast cancer. |
| **Mann-Whitney U test.** |
| **Fisher’s exact test.** |
obtain sufficient MB families because the current assay included 11 amplicons with an expanded area which lead to a decreased and varied number of reads per amplicon. For each family including over five reads, consensus sequences were mapped onto the ESR1 reference sequence using Bowtie2 ver.2.2.3, and the consensus sequence was constructed by the base accounting for over 80% at each position using SAMtools and the custom Ruby script. Insertion/deletion analysis was performed using lofreq2. Four amplified libraries of each sample were separately analyzed, and when at least one variant family was detected in all four libraries, they were considered as true mutations. Although two libraries per sample were analyzed in our last study [17], the number of libraries was increased from two to four to suppress the background errors to below 0.1% at all SNVs.

Statistics

R 3.5.1 was used for statistical processing. Fisher’s exact test was used to compare 2×2 and 2×3 groups, Mann-Whitney U test was used to compare the duration of therapy, and log-rank test was used to analyze the prognosis. P < .05 was considered significant.

Results

Development of Multiplex MB-NGS for ESR1-LBD

Figure 1 shows the scheme of 11 primers for multiplex MB-NGS that cover 96.8% (691 / 714 bp) of the whole LBD (c.928-1641) of the ESR1 gene (accession: M12674.1/AAA52399.1). Two gaps (c.1236-1242 and c.1538-1553) included neither hotspots (c.1601-1613, a.V534-D538) nor other frequent mutations such as E380Q (G1138C) and S463P (T1387C). DNA samples from plasma (2 ml) of 10 healthy controls were analyzed by multiplex MB-NGS. The median read depth of each amplicon was 156,800 (range from 69,862 to 578,168), and the median number of MB families of each amplicon was 6043 (range from 2309 to 33,266), which was sufficient to obtain a detection sensitivity of 0.1%. In conventional analysis without MB, the background errors were observed in all of the possible 2073 SNVs, with a median variant allele frequency (VAF) of 0.014% (0.000-1.317%), and 16.1% (334 variations) of them were greater than 0.1%. After MB analyses, 98.8% (2049 SNVs) of these background SNVs were completely removed, and the frequencies of all the remaining 24 (1.2%) SNVs were below 0.1% (median: 0.017%; range: 0.003-0.074%) (Figure 2). Because the current assay targeted as many as 2073 SNVs, a sensitivity test by spike-in mutations could not be performed. Thus, the detection limit of the multiplex MB-NGS assay was set at 0.1% in the following analyses based on the result that the background errors were always <0.1% at any SNVs in controls.

ESR1 Mutations in Plasma DNA from Patients with MBC

DNA from plasma (2 ml) of 54 patients with MBC was analyzed by multiplex MB-NGS for ESR1-LBD. Seventeen mutations were detected in 13 patients (24%), with VAFs ranging from 0.13 to 10.67%, including six mutations with VAF < 1% (Figure 3, Table 2). Although total sequence reads showed a great variation (range, 339,919-5,639,259) due to different sampling years, adequate MB families (from 9895 to 290,847) were obtained in all samples. Neither insertion nor deletion (In/Del) mutation was detected. The VAFs of 17 mutations obtained by multiplex MB-NGS and conventional analysis showed good agreement.
correlation with each other (Spearman correlation: \( r = 0.973 \)). Seventeen mutations included one A312V, one S463P, two L536H, one L536P, one Y537C, seven Y537S, and four D538G mutations. AF-1/2, activation function-1/2; DBD, DNA binding domain; LBD, ligand-binding domain.

### Table 2: Detailed NGS Results of 17 ESR1 Mutations

| Case | AA Change  | Nt Change  | COSMIC ID   | MB-NGS | Conventional Analysis |
|------|------------|------------|-------------|--------|-----------------------|
|      |            |            |             | Freq. (%) | Mutant Families | Total Families | Freq. (%) | Mutant Reads | Total Reads |
| #026 | L536H      | 1607 T > A | #6843697    | 4.47    | 13,003          | 290,847       | 5.46       | 308,101      | 5,639,259  |
| #188 | D538G      | 1613A > G  | #5413588    | 7.09    | 1921           | 27,083        | 7.43       | 99,014       | 1,332,645  |
| #209 | S463P      | 1387 T > C | #4771561    | 2.00    | 1428           | 71,553        | 2.48       | 100,830      | 4,059,243  |
| #390 | L536H      | 1613A > G  | #5413588    | 4.06    | 1318           | 32,498        | 4.27       | 65,885       | 1,542,075  |
| #424 | D538G      | 1613A > G  | #5413588    | 6.82    | 15,173         | 222,588       | 9.11       | 292,095      | 3,206,519  |
| #432 | S463P      | 1387 T > C | #4771561    | 3.43    | 1404           | 32,030        | 1.29       | 64,639       | 4,996,862  |
| #482 | Y537S      | 1610A > C  | #5413589    | 0.61    | 346            | 56,983        | 0.62       | 9847         | 1,596,141  |
| #508 | Y537S      | 1610A > C  | #5413589    | 0.31    | 313            | 100,469       | 0.31       | 7124         | 2,521,223  |
| #508 | Y537S      | 1610A > C  | #5413589    | 5.55    | 4256           | 76,715        | 6.06       | 106,362      | 1,755,541  |
| #564 | S463P      | 1387 T > C | #4771561    | 1.34    | 407            | 31,764        | 0.83       | 8530         | 1,033,527  |
| #630 | Y537C      | 1610A > G  | #1074637    | 0.60    | 380            | 63,338        | 1.35       | 19,169       | 1,419,018  |
| #654 | Y537S      | 1610A > G  | #5413589    | 0.87    | 553            | 63,338        | 0.60       | 12,701       | 2,114,222  |
| #744 | Y537S      | 1610A > G  | #5413589    | 5.76    | 8967           | 155,753       | 1.23       | 25,940       | 2,114,222  |
| #848 | D538G      | 1613A > G  | #6906109    | 5.80    | 5723           | 98,681        | 6.29       | 303,965      | 4,833,854  |
| #508 | Y537S      | 1610A > C  | #5413589    | 0.13    | 48             | 37,709        | 0.10       | 2463         | 2,446,431  |

\( N.A. \), not assigned in breast cancer; \( Freq. \), frequency (Freq. < 1% of conventional analysis was described with parentheses since they could not be distinguished from background errors).

Four patients had double mutations (#026, L536H / D538G; #209, S463P / D538G; #630, Y537C / S; #744, L536P / D538G). Of these four patients, three had double mutations located in the same segment for PCR amplification (#026, #630, and #744),
enabling us to analyze whether the double mutations located in the same read or not. We could demonstrate that the double mutations were exclusively located in different reads in all three patients, demonstrating that double mutations existed in different alleles. Representative results for case #26 (double mutations; L536H / D538G) are shown in Figure 4.

**ESR1 Mutation and Clinical Course**

Clinicopathological features of the patients were assessed according to the status of ESR1 mutations (Table 1). The presence or absence of adjuvant AI treatment did not affect the frequency of ESR1 mutations, but they were significantly more frequent in patients with a longer duration of AI treatment under metastatic conditions (30.2 vs. 2.9 months, P < .001) (Table 1). ESR1 mutations tended to be more frequent in patients with liver metastasis, and this trend was significant for Y537S / D538G hotspot mutations (Supplementary Table 3). All ESR1 mutations except one (non hotspot A312V mutation in patient #479) were detected after AI treatment under metastatic conditions (Supplementary Figure 1).

**Discussion**

We have developed the multiplex MB-NGS assay to detect ESR1 mutations across the entire LBD, not limited to hotspots, with a detection sensitivity of 0.1%. In fact, of the 17 mutations detected in the present study, one mutation (A312V) was located outside the hotspots and would have been missed by the dPCR assay targeting the hotspots. Another advantage of the NGS-based assay is its ability to detect the In/Del mutations since In/Del of the ESR1 gene is reportedly very rare (<1%) in breast cancer ([19], so it was highly unlikely that it would be detected in our samples.

In line with the previous reports, the patients with hotspot mutations were all treated with AIs under metastatic conditions [4,21]. No ESR1 mutation was found in the patients treated with adjuvant AI only, including those who experienced relapse during or soon after adjuvant AI treatment, indicating that ESR1 mutations exclusively develop during the use of AI for clinically evident metastatic tumors. For the two mutations outside the same segment for PCR amplification (PolyPhen2, SHT, PROVEAN, and PANTHER) suggests that this mutation is not functional.

It has been reported that ESR1 mutations are associated with liver metastasis [22-24]. Razavi's data [23] suggested that active hotspot mutations such as Y537S and D538G are more likely to occur in liver metastasis, and this trend was significant for Y537S / D538G hotspot mutations. However, the rearrangements of ESR1 gene are reportedly very rare (< 1%) in breast cancer ([19], so it was highly unlikely that it would be detected in our samples.

The biological function of this mutation must be investigated in the future, while in silico analysis (PolyPhen2, SHT, PROVEAN, and PANTHER) suggests that this mutation is not functional.

The hotspot is thought that a future study on the detection of ESR1 mutations in cell-free DNA would be better conducted with the multiplex MB-NGS assay, which, unlike dPCR, can detect not only SNV but also In/Del mutations in the whole LBD of the ESR1 gene.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.12.007.

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