Increased frequency of *ESR1* mutation in metastatic breast cancer by dosing selective estrogen receptor modulator followed by aromatase inhibitor

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Abstract. In several recent studies on metastatic breast cancer (MBC), ligand binding domain mutations of the estrogen receptor, which is coded by the *ESR1* gene, were induced by long-term endocrine therapy and resulted in acquired endocrine therapy resistance and poor outcomes. Knowledge of the association between the development of *ESR1* mutation and the clinicopathologic features may guide the decision-making process of metastatic breast cancer treatment, including endocrine therapy. The aim of the present study was to evaluate the association between the development of *ESR1* mutation and the clinicopathologic characteristics of patients with MBC. To evaluate the association between the development of *ESR1* mutation and clinicopathologic features, a cohort of 22 patients with MBC were retrospectively analyzed using next generation sequencing. In 14 of 22 patients, four mutations were detected on the metastatic site, including Tyr537Ser, Glu542Asp, Leu536Arg and Arg548Cys. Univariate analysis demonstrated that the duration of aromatase inhibitor and selective estrogen receptor modulator treatment, as well as the age of treatment initiation for early-stage breast cancer, were significantly associated with the development of *ESR1* mutation. *ESR1* mutation was identified in all five patients who received selective estrogen receptor modulators in the adjuvant setting followed by aromatase inhibitors in the metastatic setting, as well as in two of the three patients who received no selective estrogen receptor modulators in adjuvant setting followed by aromatase inhibitors in the metastatic setting. In conclusion, the results of the present study suggested that administrating adjuvant selective estrogen receptor modulator followed by aromatase inhibitor for metastasis may increase the frequency of *ESR1* mutation.

Introduction

Estrogen receptor (ER), which is encoded in the estrogen receptor 1 (*ESR1*) gene, belongs to the nuclear hormone receptor family (1) and the *ESR1* gene is located on chromosome 6 (6q25.1) and includes 8 exons (2). ER is expressed in over 60% of breast cancers (3) and consists of two activation function domains, AF1/2, a DNA binding domain and a hinge domain, and a ligand-binding domain (LBD) (4). ER functions as a ligand-dependent transcription factor, and ligand binding to the LBD leads to activation of gene transcription, resulting in breast cancer progression (5,6). Adjuvant endocrine therapy (ET) inhibiting ER-induced breast cancer progression reduces local recurrence and mortality in patients with ER-positive early breast cancer (3,7). Similarly, ET with aromatase inhibitors (AIs), selective estrogen receptor modulators (SERMs) and selective estrogen receptor degrader (SERD) serves an important role in the treatment of patients with ER-positive metastatic breast cancer (MBC) (1). However, a number of patients with ER-positive MBC have intrinsic ET resistance or acquire resistance following response to ET, and eventually almost all patients with MBC develop resistance to ET (8). ET resistance mechanisms include upregulation of the steroid receptor coactivator-3, human epidermal growth factor 2 (HER2) or nuclear factor κB and activator protein 1 (9-11). Cyclin D1 gene amplification frequently occurs in ER-positive breast cancer and overexpression of Cyclin D1 leads to ET resistance in ER-positive breast cancer (12,13). A number of previous studies indicated that LBD mutations (i.e., Tyr537Ser and Asp538Gly) in *ESR1* gene were induced by long-term ET and resulted in acquired ET resistance independently of...
estrogen levels, as well as poor outcomes in patients with MBC (14-18). Knowledge of the associations between the development of ESR1 mutation and patient clinicopathological characteristics may guide the decision-making process of MBC treatment, including ET. The aim of the present study was to evaluate the association between the development of ESR1 mutation and the clinicopathological characteristics of patients with MBC.

Materials and methods

Clinical samples. Patients with MBC who had received treatment for primary ER-positive breast cancer and were followed-up at Keio University Hospital between January 2012 and December 2015 were enrolled in this study; during that period, 24 biopsy samples from the metastatic sites were available for analysis. The inclusion and exclusion criteria were as follows: Metastatic samples with ≥100x sequence coverage on next generation sequence (NGS) were included, while samples <100x sequence coverage were excluded from the present study. Metastatic samples were evaluated by NGS, with a level of sequence coverage of >100-fold, to detect the LBD mutations of the ESR1 gene and validate these ER mutations in patients with MBC. The 24 metastatic site samples were assessed, and two were excluded from NGS due to low sequence coverage. Thus, a total of 22 metastatic samples were evaluated in the present study. The Allred score was used to assess the receptor status at metastatic sites (19). The clinical data, including the ET administered and clinical outcomes, were obtained from all patients. The study protocol and the opt-out informed consent procedure were approved by the Ethics Review Board of Keio University Hospital and conformed to the Declaration of Helsinki. Informed consent was acquired from the patients with MBC.

DNA extraction. Using the biopsy samples from the MBC patients, unstained 10-µm thick formalin-fixed paraffin embedded (FFPE) blocks were obtained. Tumor tissue was collected from the two blocks and placed in 1.5 ml microtubes. Genomic DNA was extracted using a NucleoSpin DNA FFPE XS isolation kit (Takara Bio, Inc.). DNA was quantified by Qubit 3.0 Fluorometer using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, and adjusted to a final concentration of 20 ng/µl.

Target amplification and sequencing. For target amplification of the mutation hotspot in the LBD of the ESR1 gene, PCR of the extracted genomic DNA was performed using custom primers and ligating Illumina read1 and read2 sequences (Illumina, Inc.). The second PCR primer pairs were used to ligate the Illumina adaptor and index sequence to the first PCR products. The target amplification PCR primer sequences were as follows: Forward, 5'-ACA CTC TTT CCC TAC ACG AC CGC TCT TCG ATG TTG GCT CTT CAC TAA-3' and reverse, 5'- GGT GAG ACC CCG CTG ACG TGT TTG GCT CTT CCG ATC TTA AGG CTC TAA GAG ATC T-3' (NNN NNN: Index sequence). These sequences refer to ESR1. Fig. 1 demonstrates the target amplification PCR primer pairs and the second PCR primer pairs. In a 10-µl reaction buffer that contained 5 mM deoxynucleotide triphosphate mix, 0.25 µM of each custom made primer and 0.2 µl Herculase II Fusion DNA polymerase (Agilent Technologies, Inc.), 20 ng of the genomic DNA underwent amplification for 20 cycles of 10 sec at 98˚C, 30 sec at 55˚C and 30 sec at 72˚C. The amplicon, which was 1 µl of the PCR product diluted 10 times, was marked in a second PCR with molecular indices for Illumina Miseq, using TrueSeq DNA HT Sample Kits (Illumina, Inc.). The second amplification was performed as aforementioned.

DNA libraries were formed from the second PCR products, which were purified using the Agencourt AMPure XP reagent, according to the manufacturer's instructions (Beckman Coulter, Inc.) and quantified by the method described above. The library was sequenced on the Miseq instrument on the paired-end mode with the Miseq Reagent kit v3 (Illumina, Inc.) according to the manufacturer's instructions. The sequence data were mapped to the reference human genome (hg19) using BWA aligner [version 0.7.16a-r1181; (20)], SAMtools [version 1.1; (21)] and Picard (http://sourceforge.net/projects/picard). Local alignment and quality score calibration were performed according to the Genome Analysis Toolkit (GATK) best practice (22). Single nucleotide variants were called using the 'HaplotypeCaller' tool in GATK. All variants were annotated using snpEff (23) and reviewed by the Integrative Genomics Viewer (24). Variants were filtered using dbSNP_138 (http://www.ncbi.nlm.nih.gov/projects/SNP). GRCh37.75 in the Ensembl genome browser (http://www.ensembl.org) was used as the reference genome of annotation.

Statistical analysis. Statistical analyses were performed with R commander (version 2.4-1) based on R (version 3.3.3; http://cran.r-project.org/) and with EZR, which is a modified version of the R commander (25). Among the continuous variables, age followed a normal distribution as determined by the Shapiro-Wilk test; thus, it was presented as the mean ± standard deviation and analyzed using the independent two-sample t-test. The other continuous variables were expressed as the median (range) and evaluated by the Wilcoxon rank sum test. Categorical variables were analyzed by the Fisher's exact test. The correlation between two continuous variables was evaluated by calculating Spearman's rank correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics and detection of ESR1 mutations in patients with MBC. The baseline characteristics of the 22 patients with MBC are presented in Table 1. The mean age of the patients at the start of treatment was 54 years. The TNM stage of primary breast cancer was I in 7 (32%) patients, IIa in 9 (41%) patients, IIb in 4 (18%) patients, IIIb in 1 (5%) patient and IV in 1 (5%) patient. The histologic type of primary lesion was invasive ductal carcinoma in 21 (95%) patients and invasive lobular carcinoma in 1 (5%) patient. The Progesterone receptor
(PgR) and HER2 status of the primary tumor was positive in 21 (95%) and four (18%) patients, respectively; HER2 status was unknown in one case.

As presented in Table II, ESR1 mutation at the metastatic site was observed in 14 (64%) of the 22 patients. A total of four ESR1 mutations were identified including 1610A>C, 1626G>T, 1607T>G and 1642C>T, which led to amino acid mutations Tyr537Ser in 10 (45%) patients, Glu542Asp in 2 (9%) patients, Leu536Arg in 1 (5%) patient and Arg548Cys in 1 (5%) patient, respectively. The range of allele mutation frequency was 2-99%.

ESR1 mutation and the clinical characteristics of patients with MBC. The ER Allred score of the primary tumor was positive by immunohistochemical staining in all 22 patients prior to this study (19). Of the 22 samples, 21 and 17 were positive for ER and PgR, respectively (unavailable in 1 sample). The metastatic biopsy site was the liver in 7 (32%) patients, skin in 6 (27%) patients, lymph node in 4 (18%) patients, lung in 3 (14%) patients, bone in 1 (5%) patient and muscle in 1 (5%) patient.

Among the 22 patients with MBC, including one with stage IV cancer, 19 patients received at least one ET agent.
prior to the metastatic site biopsy; ESR1 mutation was detected in 13 patients, but it was not detected in 6 patients. Of the 21 patients with MBC (excluding the patient with stage IV MBC), 16 patients received ET in an adjuvant setting, which was complete in 10 cases and incomplete in 6 cases. An incomplete adjuvant setting was defined as recurrence of breast cancer within five years of treatment. Among the 16 patients who received ET in the adjuvant setting, ESR1 mutation was detected in 2 of 6 patients who received AIs, in 8 of 9 patients who received SERMs and in 1 patient who received both AI and SERM. Between the recurrence and the biopsy of the metastatic site, 7 of 8 patients who received AIs developed ESR1 mutation. ESR1 mutation was identified in all 5 patients who received SERM in the adjuvant setting followed by AI in the metastatic setting, as well as in 2 of 3 patients who did not receive SERM in adjuvant setting followed by AI in the metastatic setting. In addition, 7 of 8 patients who had no ESR1 mutation did not receive any ET for metastasis. The patient with stage IV MBC, who was administered AIs until the biopsy of the metastatic site, developed ESR1 mutation. These results are presented in Table III.

Association between ESR1 mutation and clinicopathological characteristics in 22 patients with MBC. Considering the total period between the beginning of treatment and the biopsy of the metastatic site in 22 patients with MBC, the SERM intake period was significantly longer in patients with ESR1 mutation compared with that in patients without mutant ESR1 (49±11 vs. 63±12 years; P=0.01; Table IV). There were no significant differences in the primary TNM stage, histologic type, PgR and HER2 status of primary tumor between the two patient groups. The number of administered ETs tended to be higher in patients with ESR1 mutation compared with that in patients without ESR1 mutation, but the difference was not significant (1.5 vs. 0, respectively; P=0.06; Table IV). The age at the time of initiating treatment was associated with the SERM intake period (Spearman's rank correlation coefficient, -0.45; P=0.03; Table IV).

Discussion

In the present study on a cohort of patients with MBC, the presence of LBD mutations in the ESR1 gene was detected by targeted NGS, and its association with patient clinicopathologic characteristics was assessed. The frequency of ESR1 mutations in metastatic samples was 64% in this study and varied between 13 and 55% among published studies that also used NGS (14-16,26). Compared with previous studies,
the present study had a higher frequency of \textit{ESR1} mutations and a lower number of administered ETs. This study demonstrated that there was no association between the number of administered ET and the development of \textit{ESR1} mutation. Two previous studies have demonstrated an association between ET exposure and the prevalence of mutated \textit{ESR1} (26,27), although the number of previously administered ET was not clarified.

In the current study, the \textit{ESR1} mutations Leu536Arg, Tyr537Ser, Glu542Asp and Arg548Cys were detected. The Tyr537Ser and Arg548Cys mutations have been demonstrated to induce estrogen-independent activity of the ER, leading to ET resistance (15,28). In addition, bioinformatics analysis has indicated that an Arg548Cys mutation in the ER is deleterious (29). Similarly, amino acid mutations in Leu536 have been reported to increase the estrogen-independent activity of the ER (30), and Toy \textit{et al} (15) identified the Leu536Arg mutation in the ER, but they did not investigate its function. The impact of the Glu542Asp alteration on the ER function remains unknown and further investigation is needed (31). Therefore, the detected ER mutations in this study, with the exception of Glu542Asp, may induce a ligand-independent ER activation resulting in ET resistance. It is important to determine the changes of \textit{ESR1} alterations between primary and metastatic tumor; however, in this study, the \textit{ESR1} mutations of the primary lesion were not investigated because primary breast cancers have very rare \textit{ESR1} mutation, which has been reported in previous studies, including The Cancer Atlas data (32-34).

A number of previous studies on patients with MBC have demonstrated that compared with wild-type \textit{ESR1}, \textit{ESR1} mutation led to worse progression-free survival (PFS) and overall survival (OS) (35,36). However, in the present study, \textit{ESR1} mutation had no adverse impact on the outcomes of patients with MBC (data not shown). The small cohort used in the present study limited the statistical power to assess the impact on the outcomes of patients with MBC.

This may be due to the Glu542Asp mutation, which was detected in 2 patients with MBC in this study, having no negative effect on the patients' outcomes. The present study revealed that prolonged AI treatment for metastasis had a significant impact on the development of \textit{ESR1} mutation and that patients with MBC who received AIs in an adjuvant setting exhibited low rates of \textit{ESR1} mutation. A number of previous studies reported that mutated \textit{ESR1} rarely occurred during adjuvant therapy with AI, but its prevalence was high.

### Table III. \textit{ESR1} mutations and the clinical data of 22 patients with metastatic breast cancer.

| Case no. | Age, years\textsuperscript{a} | Mutations | Allred of E/P\textsuperscript{b} | Biopsy site | Adjuvant ET (months, status) | ET after recurrence to biopsy |
|----------|------------------|-----------|------------------|-------------|-----------------------------|-----------------------------|
| 1        | 74               | Yes       | 7/0              | Skin        | AI (21, incomplete)         | None                        |
| 2        | 48               | Yes       | 8/8              | Liver       | SERM (60, complete)         | AI, SERM, AI                |
| 3        | 51               | Yes       | 7/7              | Skin        | AI (7), SERM (53, complete) | None                        |
| 4        | 35               | Yes       | 8/8              | LN          | None                        | None                        |
| 5        | 30               | Yes       | 7/5              | LN          | None                        | AI, SERM, AI, SERD          |
| 6        | 56               | Yes       | Unavailable      | Bone        | AI (60, complete)           | AI                          |
| 7        | 54               | Yes       | 8/7              | Skin        | SERM (60, complete)         | None                        |
| 8        | 47               | Yes       | 7/0              | Liver       | SERM (22, incomplete)       | AI, SERD                    |
| 9        | 54               | Yes       | 8/6              | Skin        | SERM (60, complete)         | AI                          |
| 10       | 39               | Yes       | 8/8              | Liver       | SERM (24, incomplete)       | AI, AI                      |
| 11       | 51               | Yes       | 8/8              | Liver       | SERM (24, complete)         | None                        |
| 12       | 57               | Yes       | 8/7              | Liver       | SERM (60, complete)         | AI                          |
| 13       | 42               | Yes       | 8/5              | Skin        | SERM (37, incomplete)       | None                        |
| 14       | 46               | Yes       | 8/8              | Liver       | None                        | AI, AI                      |
| 15       | 74               | No        | 8/8              | Skin        | AI (60, complete)           | None                        |
| 16       | 75               | No        | 8/8              | Muscle      | None                        | None                        |
| 17       | 58               | No        | 8/6              | LN          | AI (60, complete)           | None                        |
| 18       | 39               | No        | 8/0              | LN          | SERM (23, incomplete)       | None                        |
| 19       | 59               | No        | 8/8              | Lung        | None                        | AI, SERM                    |
| 20       | 63               | No        | 7/7              | Lung        | AI (58, incomplete)         | None                        |
| 21       | 60               | No        | 8/0              | Liver       | AI (60, complete)           | None                        |
| 22       | 74               | No        | 8/5              | Lung        | None                        | None                        |

\textsuperscript{a}Age at the start of treatment. \textsuperscript{b}Allred score of E/P at metastasis. The patient had stage IV breast cancer. Incomplete adjuvant setting is defined as the recurrence of breast cancer during adjuvant treatment. E/P, estrogen receptor/progesterone receptor; \textit{ESR1}, estrogen receptor 1; ET, endocrine treatment; AI, aromatase inhibitor; SERM, selective estrogen receptor modulator; SERD, selective estrogen receptor degrader; LN, lymph node; PgR, progesterone receptor.
TAKESHIMA et al: INCREASED FREQUENCY OF ESRI MUTATION IN MBC

Table IV. Association between estrogen receptor 1 gene mutation and clinicopathologic data in 22 patients with metastatic breast cancer.

A. According to the total duration from the beginning of treatment to biopsy of the metastatic site (n=22)

| Variable     | Mutation (+) | Mutation (-) | P-value |
|--------------|--------------|--------------|---------|
| AI           | 15 (0-83)    | 31 (0-60)    | 0.92    |
| SERM         | 26 (0-60)    | 0 (0-23)     | 0.01    |
| AI + SERM    | 60 (0-143)   | 41 (0-60)    | 0.13    |

B. According to the total duration from recurrence to biopsy of the metastatic site (n=21)

| Variable     | Mutation (+) | Mutation (-) | P-value |
|--------------|--------------|--------------|---------|
| AI           | 5 (0-83)     | 0 (0-3)      | 0.04    |
| SERM         | 0 (0-27)     | 0 (0-7)      | 0.83    |
| AI + SERM    | 5 (0-83)     | 0 (0-10)     | 0.05    |

C. According to clinicopathological characteristics (n=22)

| Variable                          | Mutation (+) | Mutation (-) | P-value |
|-----------------------------------|--------------|--------------|---------|
| Age at the start of treatment, years | 49±11        | 63±12        | 0.01    |
| Primary TNM stage                  |              |              |         |
| I                                 | 4            | 3            |         |
| IIA                               | 5            | 4            |         |
| IIIB                              | 3            | 1            |         |
| IIIA                              | 0            | 0            |         |
| IIIB                              | 1            | 0            |         |
| IIC                               | 0            | 0            |         |
| IV                                | 1            | 0            |         |
| Histological type                 |              |              |         |
| Invasive ductal                   | 13           | 8            |         |
| Invasive lobular                  | 1            | 0            | >0.99   |
| PgR status of the primary tumor   |              |              |         |
| Positive                          | 13           | 8            |         |
| Negative                          | 1            | 0            | >0.99   |
| HER2 status of the primary tumor  |              |              |         |
| Positive                          | 2            | 2            |         |
| Negative                          | 11           | 6            | 0.62    |
| Total number of administered ET   | 1.5 (0-3)    | 1 (0-1)      | 0.06    |
| The number of ET from recurrence to biopsy | 1 (0-3) | 0 (0-2) | 0.10 |
| Spearman’s rank correlation       |              |              |         |
| Age at the start of treatment vs. | -0.45        |              | 0.03    |
| The total duration of SERM until biopsy of the metastatic site | | | |

*Excluding SERD regimen. Values are expressed as the median (range) or the mean ± standard deviation and analyzed by Wilcoxon rank sum test, independent samples Student’s t-test or Fisher’s exact test. AI, aromatase inhibitor; SERM, selective estrogen receptor modulator; TNM, Tumor-Node-Metastasis; PgR, progesterone receptor; ET, endocrine treatment.

during recurrence treatment with AI (17,35). The results of the present study appeared to support these studies. Previous studies have also demonstrated the superior effects of fulvestrant on the PFS and OS compared with those of anastrozole in patients with endocrine-sensitive MBC (37,38). Furthermore, the addition of palbociclib, a CDK4/6 inhibitor,
to letrozole or fulvestrant improved the PFS in patients with MBC (39,40). Of note, it was reported that palbociclib combined with fulvestrant improved the PFS irrespective of the ESR1 mutation status in patients with MBC (41) and that palbociclib plus letrozole did not prevent the development of ESR1 mutation in a small cohort of patients with MBC who received the combination treatment (42). These results suggest that the assessment of dynamic changes of the ESR1 mutation status using minimally invasive procedures such as liquid biopsy in patients with MBC who receive CDK4/6 inhibitors may be of importance for investigating acquired resistance to these drugs.

The results of the present study demonstrated that the total period of SERM treatment was associated with the emergence of ESR1 mutation. To the best of our knowledge, the effects of SERM on ESR1 mutation have not been fully clarified to date. Among patients with MBC who received tamoxifen alone, ESR1 mutation was not detected in all 22 patients in a study by Schiavon et al (17), but it was detected in 4 out of 11 patients in a study by Takeshita et al (43). The present study demonstrated that the frequency rate of ESR1 alteration in patients who received adjuvant-SERM followed by metastatic AI treatment was higher compared with that in patients who received no adjuvant-SERM followed by metastatic AI. The association of SERM with ESR1 mutation that was identified in this study may be explained by the finding that most of the patients with mutated ESR who received AIs for metastasis had been administered SERMs in an adjuvant setting. Administration of SERM in an adjuvant setting may have been used as a compelling indication for the use of AI for metastasis; therefore, SERM for adjuvant setting, followed by AI for metastasis, may increase the frequency of ESR1 mutations. However, this result needs to be clarified and verified in a future study.

The present study revealed that age at the time of treatment initiation for breast cancer was significantly associated with the development of ESR1 mutation and the total duration of SERM treatment. These associations may be due to the premenopausal status of the majority of patients with MBC who received SERM in an adjuvant setting and AI in a metastatic setting, leading to the subsequent development of ESR1 mutation. To the best of our knowledge, no previous studies have demonstrated an association between age at the time of treatment initiation for breast cancer and the occurrence of ESR1 gene mutation.

This study had several limitations, including the retrospective design using a small cohort from a single institute. In addition, the effects of SERD on ESR1 mutations were not analyzed, as only three patients with MBC received SERD, and no multivariate analysis was performed due to the small cohort. The small cohort of this study limited the statistical power to assess the association between ESR1 mutation and clinicopathological features in patients with MBC.

In conclusion, the results of the present study demonstrated that SERM in an adjuvant setting followed by AI for metastasis may increase the frequency of ESR1 mutation, and that age at the time of treatment onset for breast cancer may be significantly associated with the development of ESR1 mutation. Further studies are needed to confirm and validate these findings.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
KT, TH, TS, MT and YK conceived and designed this study. HM, AN and TY performed the experiments and collected clinical data from the patients. KT analyzed data and drafted the manuscript. All authors revised the manuscript and approved the final version.

Ethics approval and consent to participate
The study protocol, including the opt-out informed consent procedure, was approved by the Ethics Review Board of Keio University Hospital (approval no. 20150439) and conformed to Declaration of Helsinki.

Patient consent for publication
Not applicable.

Competing interests
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