Comparison of ESR1 Mutations in Tumor Tissue and Matched Plasma Samples from Metastatic Breast Cancer Patients

Takashi Takeshita*, Yutaka Yamamoto*, Mutsuko Yamamoto-Ibusuki†, Mai Tomiguchi*, Aiko Sueta*, Keiichi Murakami*, Yoko Omoto*, and Hirotaka Iwase*

*Department of Breast and Endocrine Surgery, Graduate School of Medical Science, Kumamoto University, Honjo, Chuo-ku, Kumamoto, 860-8556, Japan; †Department of Molecular-Targeting Therapy for Breast Cancer, Kumamoto University Hospital, Honjo, Chuo-ku, Kumamoto, 860-8556, Japan; ‡Department of Endocrinological and Breast Surgery, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji Agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto, 602-0841, Japan

Abstract

BACKGROUND: ESR1 mutation in circulating cell-free DNA (cfDNA) is emerging as a noninvasive biomarker of acquired resistance to endocrine therapy, but there is a paucity of data comparing the status of ESR1 gene in cfDNA with that in its corresponding tumor tissue. The objective of this study is to validate the degree of concordance of ESR1 mutations between plasma and tumor tissue. METHODS: ESR1 ligand-binding domain mutations Y537S, Y537N, Y537C, and D538G were analyzed using droplet digital PCR in 35 patients with metastatic breast cancer (MBC) (35 tumor tissue samples and 67 plasma samples). RESULTS: Of the 35 paired samples, 26 (74.3%) were concordant: one patient had detectable ESR1 mutations both plasma (ESR1 Y537S/Y537N) and tumor tissue (ESR1 Y537S/Y537C), and 25 had WT ESR1 alleles in both. Nine (25.7%) had discordance between the plasma and tissue results: five had mutations detected only in their tumor tissue (two Y537S, one Y537C, one D538G, and one Y537S/Y537N/D538G), and four had mutations detected only in their plasma (one Y537S, one Y537N, and two Y537S/Y537N/D538G). Furthermore, longitudinal plasma samples from 19 patients were used to assess changes in the presence of ESR1 mutations during treatment. Eleven patients had cfDNA ESR1 mutations over the course of treatment. A total of eight of 11 patients with MBC with cfDNA ESR1 mutations (72.7%) had the polyclonal mutations. CONCLUSION: We have shown the independent distribution of ESR1 mutations between plasma and tumor tissue in 35 patients with MBC.

Introduction

Endocrine therapy resistance is one of the leading problems in patients who are estrogen receptor (ER) positive and who have metastatic breast cancer (MBC). Extensive tumor genotyping studies revealed that ESR1 nonsynonymous ligand-binding domain (LBD) mutations in a “hot spot” confined to Tyr537 and Asp538 become clear as a biomarker of endocrine therapy resistance in patients with MBC [1–6]. These recurring ESR1 mutations allow activation of ER-dependent transcription and proliferation due to the conformational change of ER in the absence of ligand, and cause the resistance to ER antagonists [3,4,7].
Tumor tissue is the gold standard specimen for tumor genotyping. Recent developing methods of gene alterations’ analysis from fragmented alleles have made it possible to detect rare gene alterations of cell-free DNA (cfDNA) in blood, such as beads, emulsion, amplification, magnets technology [8], digital polymerase chain reaction (dPCR) technology [9], pyrophosphorolysis-activated polymerization [10] or tagged-amplicon deep sequencing [11]. Numerous groups have examined the presence of ESR1 mutations in cfDNA of patients with MBC and have clarified the utility of them as a biomarker for disease monitoring, predicting prognosis, and therapeutic decision-making [12–16].

The main drawback to the use of cfDNA as a surrogate for tissue is a high degree of variability in the concordance rate between the gene alterations detectable in tumor tissues and those in their corresponding plasma, but a few groups reported the various concordance rates of ESR1 mutations between them. Schiavon et al. reported there was 97% plasma, but a few groups reported the various concordance rates of alterations detectable in tumor tissues and those in their corresponding reaction (dPCR) technology [9], pyrophosphorolysis-activated polymerization [10] or tagged-amplicon deep sequencing [11]. Numerous groups have examined the presence of ESR1 mutations in cfDNA of patients with MBC and have clarified the utility of them as a biomarker for disease monitoring, predicting prognosis, and therapeutic decision-making [12–16].

The objective of this study was to validate the distribution of ESR1 mutations between plasma and tumor tissue. We performed droplet dPCR (ddPCR), with a number of reports highlighting its superior accuracy [18,19], for each representative four ESR1 mutations (i.e., ESR1 Y537S, Y537N, Y537C, and D538G, which cover more than 80% of ESR1 mutations associated with acquired resistance to endocrine therapy [3–5]) on matching tumor tissue and plasma samples from 35 patients with MBC. In addition, we performed an exploratory analysis of the change of ESR1 mutations in longitudinal plasma samples, collected at more than two time points during the clinical course, from 19 patients during treatment.

Methods

Patients and Breast Cancer Tissue and Plasma Samples

A total of 35 patients (35 tumor tissue samples and 67 plasma samples) with MBC, treated at Kumamoto University Hospital between 2007 and 2014, were enrolled in this study. Cases were selected if an archival plasma sample and its corresponding tumor tissue were available. Informed consent was obtained from all patients before biopsy or surgery. The Ethics Committee of Kumamoto University Graduate School of Medicine (Kumamoto, Japan) approved the study protocol. The treatment of patients with MBC was performed in accordance with the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology [20]. Basically, patients with MBC were assessed monthly for clinical response at the Kumamoto University Hospital. Progressive disease was defined as the identification of positive spots by physical examination and/or by imaging diagnosis during the follow-up period.

Sample Preparation

Genomic DNA from formalin fixed, paraffin embedded tissue samples that included more than three tissue cores was extracted as cfDNA using the All Prep DNA/RNA Mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions. ER positive tumor cells were captured by laser microdissection (LMD) using a Leica LMD 7000 (Leica Microsystems K.K., Tokyo, Japan), referring to the ER-stained slide. The dissected tissues were incubated with a PicoPure® DNA Extraction Kit (Life Technologies, Tokyo, Japan) as described elsewhere [21]. After heat inactivation, the sample and its corresponding tumor tissue were available. Informed consent was obtained from all patients before biopsy or surgery. The samples from 35 patients with MBC. In addition, we performed an exploratory analysis of the change of ESR1 mutations in longitudinal plasma samples, collected at more than two time points during the clinical course, from 19 patients during treatment.

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### Analysis of ESR1 Mutations by ddPCR

ddPCR assay was carried out in the same sample twice using the QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [21]. PCR data were quantified using QuantaSoft™ software (Bio-Rad Laboratories), and the results are expressed as a percentage of mutant to total (mutant + wild type) for each tumor tissue sample and as copies per microliter of mutant DNA for each plasma sample. Our ddPCR method has been optimized by comparative analysis of a dilution series of each synthetic ESR1 mutant oligonucleotide as reported previously [14,21]. All samples were compared with the ESR1 wild-type (WT) molecule and each ESR1 mutant molecule as positive control. A water-only (no template) control was run in parallel for each ddPCR reaction as negative control. According to tDNA, the cutoff level was 11.2% in ESR1 Y537S, 15.3% in Y537N, 5% in Y537C, and 7.5% in D538G, respectively, as described previously [21]. According to plasma cfDNA, a mutation was considered positive with more than three ESR1 mutant droplets because this assay could detect as few as three copies of the mutant allele in an abundance of WT DNA (data not shown). In the longitudinal analysis, changes of cfDNA ESR1 mutations were defined as whether they existed or not during treatment.

### Probes and Primers

The ddPCR assay for the detection of the representative four ESR1 LBD mutations in ESR1 exon 8, ESR1 Y537S, Y537N, Y537C, and D538G consisted of a pair of primers and two TagMan minor groove binding probes, respectively, as described previously [21].

### Immunohistochemistry

Immunohistochemical staining was carried out on 4-μm-thick tumor sections. Serial sections were prepared from selected blocks and float-mounted on adhesive-coated glass slides for estrogen receptor alpha (ERα), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Primary antibodies, their visualization methods, and their evaluation were according to previously described methods [22].

### Statistical Analysis

The chi-square test or Fisher’s exact test was used to assess baseline differences between binary variables. The nonparametric Mann–Whitney U test was adopted for statistical analysis of the associations of total alleles in plasma with clinicopathological factors. Differences were considered significant when a value of P < .05 was obtained. All
statistical analyses were two-sided and were performed using JMP software version 10.0.1 for Windows (SAS Institute Japan, Tokyo, Japan).

**Results**

A total of 35 patients with MBC (35 tumor tissue samples and 67 plasma samples) were enrolled in this study. All tumor tissue and plasma samples contained sufficient DNA for this study. The median concentration of fragmented alleles in plasma was 117 pg/μL (range, 14–1018), and it varied by 70-fold. There was no statistically significant correlation of total allele concentration in plasma with clinicopathological factors (Table S1). The demographics and baseline characteristics of patients with MBC are presented in Table 1. The median age of the patients at blood and corresponding tumor tissue biopsy was 56 years (range, 31–84). Of the 35 metastatic tumor tissue samples, 12 (34.3%) were from skin, 9 (25.7%) were from lymph nodes, six (17.1%) were from ipsilateral breast tumor recurrence, three (8.6%) were from bone, two (5.7%) were from lung, and one sample each (2.9%) was from liver, brain, and ovary. Of the 67 plasma samples, 35 plasma samples were drawn in the biopsy of the tumor tissue and 32 plasma samples were drawn before or after tumor tissue biopsy (two points from a total of 9 patients (47.4%), three points from a total of seven patients (36.8%), and four points from a total of three (15.8%) of 19 patients with MBC). A total of 33 of 35 patients (94.3%) had been treated with hormonal therapy and a total of 17 of 35 patients (48.6%) were treated with hormonal and chemotherapy. In addition, three patients were treated simultaneously with targeted therapy using trastuzumab. A total of two of 35 MBC cases (5.7%) had not received any treatment before recurrence for the following reasons: one had history of cerebral infarction and the other had microinvasive disease. Both these patients were treated with endocrine therapy after recurrence. The median duration of follow-up was 146 months (range, 15–284 months).

We performed a ddPCR assay to screen representative four ESR1 mutations in MBC tissues and plasma. Six of 35 patients (17.1%) had detectable mutant ESR1 alleles in their tumor tissue samples and five of 35 patients (14.3%) had detectable mutant ESR1 alleles in corresponding plasma samples. Correlation of ttDNA and cfDNA ESR1 mutations with clinicopathological parameters in 35 patients with MBC is summarized in Table 2. The presence of cfDNA ESR1 mutations was marginally associated with higher ERα immunostaining \((P = .093)\). All five patients with cfDNA ESR1 mutations were previously treated with AIs and more than two kinds of endocrine drugs. In the analysis of corresponding MBC tumor tissue samples,
no relationship could be found between the presence of $ESR1$ mutation and clinicopathological factors.

Comparison of the plasma and tissue results for these 35 patients revealed concordance of results in 26 of 35 (74.3%) patients: one patient had detectable $ESR1$ mutations both plasma ($ESR1$ Y537S/Y537N) and tumor tissue ($ESR1$ Y537S/Y537C), and 25 had WT $ESR1$ alleles in both their plasma and tumor tissues. Nine of 35 patients had discordance between the plasma and tissue results: five had mutations detected only in their tumor tissue (two Y537S, one Y537C, one D538G, and one Y537S/Y537N/D538G), and four had mutations detected only in their plasma (one Y537S, one Y537N, and two Y537S/Y537N/D538G) (Figure 1A).

Additionally, longitudinal plasma samples, collected at more than two time-points of the clinical course, from 19 patients were used to assess changes in the presence of $ESR1$ mutations during treatment (Figure 1B). In 12 patients, plasma samples were obtained before tumor tissue biopsy, and in seven of these patients, the samples were collected after tumor tissue biopsy. Of 29 patients with ttDNA $ESR1$ WT, 8 patients (27.6%) did not have the changes in the status of $ESR1$ cfDNA during treatment (six patients (26.7%) had cfDNA $ESR1$ WT and two patients (6.9%) had cfDNA $ESR1$ mutations), but 8 patients (27.6%) had the changes in the presence of cfDNA $ESR1$ mutations during treatment (cfDNA $ESR1$ mutations disappeared in four patients (13.8%) and newly appeared in four patients (13.8%)). Of six patients with ttDNA $ESR1$ mutations, two patients (33.3%) have not had any cfDNA $ESR1$ mutations, but one patient (16.7%) had acquired cfDNA $ESR1$ mutations during treatment. Clinical details of 11 ER-positive MBC patients with cfDNA $ESR1$ mutations during treatment are shown in Table S2. A total of eight (72.7%) of 11 MBC patients with cfDNA $ESR1$ mutations had the polyclonal mutations over the course of treatment. Aside from case 9, all patients were treated with Al before tumor tissue biopsy. Case 34 lost $ESR1$ mutations (Y537S/N, D538G) in both tumor tissue and paired plasma after treatment with AI.

**Figure 1.** A, Chart showing the percentage of the correlation in the status of $ESR1$ gene between tumor tissue and plasma in this cohort. Six of 35 patients (17.1%) had detectable mutant $ESR1$ alleles in their tumor tissue samples and 5 of 35 patients (14.3%) had detectable mutant $ESR1$ alleles in corresponding plasma samples. Comparison of the plasma and tissue results for these 35 patients revealed concordance of results in 26 of 35 patients (74.3%). Nine of 35 patients had discordance between the plasma and tissue results. B, Chart showing the percentage of the change in the status of cfDNA $ESR1$ gene in patients with ttDNA $ESR1$ WT (upper) and in patients with ttDNA $ESR1$ mutations (lower). Longitudinal plasma samples, collected at more than two time points during the clinical course, from 19 patients were used to examine changes in the presence of $ESR1$ mutations during treatment. In 12 patients, the plasma samples were collected before tumor tissue biopsy, and in seven of them, the samples were collected after tumor tissue biopsy. Of 29 patients with ttDNA $ESR1$ WT, eight patients (27.6%) did not have changes in the status of $ESR1$ cfDNA during treatment, but 8 patients (27.6%) had changes in the presence of cfDNA $ESR1$ mutations during treatment. Of six patients with ttDNA $ESR1$ mutations, two patients (33.3%) did not have any cfDNA $ESR1$ mutations, but 1 patient (16.7%) acquired cfDNA $ESR1$ mutations during treatment. Abbreviations: WT, wild-type; cfDNA, cell-free DNA; ttDNA, tumor tissue DNA; Mu, mutation.
Discussion

In this study, we performed ddPCR for each of representative four ESR1 mutations (i.e., ESR1 Y537S, Y537N, Y537C, and D538G) on cell-free plasma samples from 35 MBC patients with known distribution of all four ESR1 mutations in metastatic lesions. A total of 17.1% (6/35) patients had detectable mutant ESR1 alleles in their tumor tissue samples and 83.3% (5/6) patients with ESR1 mutations were previously treated with aromatase inhibitors and more than two kinds of endocrine drugs. There were 14.3% of patients (5/35) who had detectable mutant ESR1 alleles in paired plasma samples and all five patients with ESR1 mutations were previously treated with AIs and more than two kinds of endocrine drugs (Table 2), with a distribution of mutations that was less frequent than that of previously published data [4,5,13,23].

The LBD in ESR1 gene can acquire polyclonal mutations [24]. We observed a case with multiple ESR1 mutations (Y537S/Y537N/D538G) in the same tumor, but two cases with multiple ESR1 mutations had polyclonal mutations over the course of treatment (Table S2), possibly reflecting differential response of individual ESR1 mutations to treatments [15].

Comparison of the plasma and tissue results for these 35 patients revealed concordance of results in 26 of 35 patients (74.3%), but 9 of 35 patients had discordance between the plasma and tissue results (Figure 1A). It is conceivable that the variability in the concordance rate between tumor tissue and plasma might be explained by the following three reasons. First, the substantial selection pressure due to endocrine therapies causes intertumoral and/or intratumoral heterogeneity, which may miss subclonal populations in a given metastatic lesion [25]. A prior report demonstrated differences in ESR1 mutation status between two metastatic sites within the same patient [7]. Second, it is regarded that as a cause of discordance between them, cfDNA itself can show the integration of somatic mutations from distinct populations of tumor cells and different metastases. Finally, the cfDNA is fragmented DNA reflecting the degradation of DNA following apoptosis and/or necrosis [26]. Thus, the quantity and quality of cfDNA is dependent on a functional disorder of infiltrating phagocytes or the location, size, and vascularity of the tumor [27], perhaps generating the variability in the concordance rate between tumor tissue and plasma. Longitudinal plasma samples from 19 patients were used to examine changes in the presence of ESR1 mutations during treatment (Figure 1B). Of 29 patients with ctDNA ESR1 WT, 8 patients (27.6%) did not have changes in the status of ESR1 gene during treatment, but 8 patients (27.6%) had changes in the presence of ESR1 mutations during treatment (cfDNA ESR1 mutations disappeared in four patients (13.8%) and newly appeared in four patients (13.8%)). Of six patients with ctDNA ESR1 mutations, two patients (33.3%) did not have any ESR1 mutations, but one patient (16.7%) had acquired ESR1 mutations during treatment.

The current study has limitations. This was a retrospective, single-institute study with a relatively small patient cohort. In addition, this cohort was very heterogeneous as it was not only based on the clinicopathological factors but also based on the treatment arms.

Conclusions

We have shown the independent distribution of ESR1 mutations between plasma and tumor tissue in 35 patients with MBC. As more data regarding the presence of actionable genomic alterations in breast cancer tissue and paired blood become available, it would be possible to clarify the differences in the clinical significance and utility of the genomic alterations in each sample.

Disclosure Statement

All the authors declare that they have no actual, potential, or perceived conflicts of interest with regard to the manuscript submitted for review.

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References

[1] Fuqua SA, Fitzgerald SD, Alleed DC, Elledge RM, Nawaz Z, McDonnell DP, O’Malley BW, Greene GL, and McGuire WL (1992). Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res 52(2), 483–486.
[2] Weis KE, Ekena K, Thomas JA, Lazennec G, and Kazenellenhogen B (1996). Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Mol Endocrinol 10(11), 1388–1398.
[3] Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, Kyaleza-Sundaram S, Wang R, Ning Y, and Hodges L, et al (2013). Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet 45(12), 1446–1451.
[4] Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, Li Z, Gala K, Fanning S, and King TA, et al (2013). ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet 45(12), 1439–1445.
[5] Jeshlohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM, Ferrer-Lozano J, Perez-Fidalgo JA, Cristofanilli M, and Gomez H, et al (2014). Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res 20(7), 1757–1767.
[6] Oesterreich S and Davidson NE (2013). The search for ESR1 mutations in breast cancer. Nat Genet 45(12), 1415–1416.
[7] Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Drir A, Soussan-Gutman L, Jeshlohn R, Yelensky R, Brown M, Miller VA, and Sarid D, et al (2013). D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res 73(23), 6856–6864.
[8] Li M, Diehl F, Dressman D, Vogelstein B, and Kinzler KW (2006). BEAMing up for detection and quantification of rare sequence variants. Nat Methods 3(2), 95–97.
[9] Vogelstein B and Kinzler KW (1999). Digital PCR. Proc Natl Acad Sci U S A 96(16), 9236–9241.
[10] Liu Q and Sommer SS (2000). Pyrophosphorylisis-activated polymerization (PAP): application to allele-specific amplification. Biotechniques 29(5), 1072–1076 [1078, 1080 passim].
[11] Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DW, Kaper F, Dawson SJ, Piskorz AM, Jimenez-Linan M, and Bentley D, et al (2012). Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med 4(136), 136ra168.
[12] Garcia-Murillas I, Schiavon G, Weigtel B, Ng C, Hrebien S, Cutts RJ, Cheang MC, Osnin P, Nerurkar A, and Kozarewa I, et al (2015). Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 7(302), 302ra133.
[13] Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, Fenwick K, Kozarewa I, Lopez-Knowles E, and Rabas R, et al (2015). Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med 7(313), 313ra182.
[14] Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Inao T, Sueta A, Fujiwara S, Omoto Y, and Iwase H (2016). Clinical significance of monitoring ESR1 mutations in circulating cell-free DNA in estrogen receptor positive breast cancer patients. Oncotarget.
Chandarlapaty S, Chen D, He W, Sung P, Samoila A, You D, Bhatt T, Patel P, Voi M, and Gnant M, et al (2016). Prevalence of ESR1 Mutations in Cell-Free DNA and Outcomes in Metastatic Breast Cancer: A Secondary Analysis of the BOLERO-2 Clinical Trial. *JAMA Oncol* **2**(10), 1310–1315.

Fribbens C, O’Learny B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, Cristofanilli M, Andre F, Loi S, and Loibl S, et al (2016). Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *J Clin Oncol* **34**(25), 2961–2968.

Chu D, Paoletti C, Gersh C, VanDenBerg D, Zabransky D, Cochran R, Wong HY, Valda Toro P, Cidado J, and Croessmann S, et al (2015). ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. *Clin Cancer Res*.

Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, Dunning MJ, Gale D, Forshew T, and Mahler-Araujo B, et al (2013). Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* **368**(13), 1199–1209.

Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, and Tewari M (2013). Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* **368**(13), 1199–1209.

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Matthews N, Stewart A, and Tarpey P, et al (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med* **366**(10), 883–892.

Diaz Jr LA and Bardelli A (2014). Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* **32**(6), 579–586.