Original Article

Evaluation of Sanger Sequencing Method for ESR1 Mutation Detection in Primary Breast Tumor

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Abstract

Objective: Exon 8 estrogen receptor 1 (ESR1) mutations in the ligand-binding domain play an important role in mechanisms of hormonal therapy resistance in breast cancer. Identification of ESR1 mutations is very important in determining the appropriate steps of therapy. In this study, we evaluate Sanger sequencing technique to detect the ESR1 mutation. **Methods:** This retrospective study was conducted using 49 advanced breast cancer patients with estrogen receptor positive. Deoxyribonucleic acid (DNA) material was extracted from primary breast tumor samples. Exon 8 ESR1 gene mutation was analyzed by Sanger sequencing method using BigDye Direct Sequencing Kit (Applied Biosystem) with gBlock synthesis gene fragment (Integrated DNA Technologies) D538G as a positive control. **Results:** The mean age of patients was 46.14 (±9.6) years, and 61.2% were in stage 4. There are no exon 8 ESR1 gene mutations detected in 49 primary tumor samples, whereas the gBlock-positive control showed base substitution in 1613A>G (D538G) indicating the success of sequencing reaction. **Conclusion:** Sanger sequencing has failed to detect ESR1 mutation in primary tumor breast samples. Other advanced molecular techniques should be performed for diagnosis of primary breast tumors.

Keywords: Breast cancer, ESR1 mutation, exon 8, Sanger sequencing

Introduction

The mortality from breast cancer has been at an all-time high over the past 10 years at Dharmais Hospital National Cancer Center in Indonesia. The majority of breast cancer patients diagnosed in Indonesia are already in Stage 3 or 4, which results in low life expectancy.[1] One type of therapy that can be an effective treatment for these cases is hormonal therapy. Hormonal therapy is reported to be able to increase survival rates for terminal stages of breast cancer patients with lower side effects than chemotherapy.[2] Various types of hormonal therapies have been used to target and control estrogen in breast cancer patients. These types of hormonal therapies include selective estrogen receptor (ER) modulators, such as tamoxifen; selective ER downregulators, such as fulvestrant; and aromatase inhibitors, such as letrozole, anastrozole, and exemestane. Other methods include the group of gonadotropin-releasing hormone analogs, such as goserelin, antiprogestin, antiandrogen, or surgical removal of the ovaries (oophorectomy).[3-5]

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Despite the fact that approximately 50% of patients with ER-positive (ER^+) breast cancer benefit from adjuvant hormonal treatment, *de novo* or acquired resistance often emerges after prolonged exposure and became a major clinical problem.[6-8] Several mechanisms of resistance to hormonal therapies have been proposed including downregulation of ER expression, deregulation of ER coregulators, posttranslational modification of ER, and crosstalk with growth factor signaling pathways.[8] Recently, the mutations in the estrogen receptor gene (ESR1) have started to emerge as another potential mechanism implicated in acquired hormonal resistance. The majority of these mutations are located within ligand-binding domain, create a ligand-free constitutively activated ER,[9] is associated with a worse outcome, and hence, it is currently

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evaluated as a potential biomarker to guide the therapeutic decision.[10] The reported incidence of the mutation was as low as <1% in primary tumors and as high as 11%–55% in metastatic ER+ breast cancer.[9] Approximately, 12 ESR1 point mutations have been described, with a hotspot confined to codons 537 and 538 in exon 8.[11] The mutation of D538G in the ESR1 gene shows a ligand-binding domain conformational change in the ER that increases proliferation and causes resistance to tamoxifen.[12] When linked to patient survival, the D538G mutation in breast cancer patients has a low survival time (25.99 months) compared to breast cancer patients with normal ESR1 (32.1 months), whereas breast cancer patients with Y537S mutations have a lower survival time (19.98 months) despite hormonal therapy in the form of exemestane and everolimus.[13] This evidence shows that ESR1 gene mutations need to be investigated because of their importance in choosing an effective therapy for breast cancer patients with positive receptor hormones. In general, the examination of ESR1 genes is carried out through next-generation sequencing (NGS) technology to obtain a comprehensive picture of the gene mutation. This is because of the ability of NGS technology to read base sequences in large numbers (high-throughput data) with high sensitivity (75%–99%).[14] However, the NGS analysis is less cost-effective for a small number of sequencing targets (one to twenty targets) and more time-consuming for sample preparation.[15] Although most studies have demonstrated that ESR1 mutation is very rare in primary breast tumor, genetic profiling for ESR1 in our population has yet not been confirmed despite majority of our samples are primary breast tumor tissue. In this study, we attempted to detect ESR1 mutation using Sanger sequencing method. This method is considerably faster and more cost-effective for low numbers of sequencing targets. Moreover, it has a familiar laboratory workflow and more likely to be applied as a molecular examination in the clinical settings.[16] The detection of ESR1 mutations using Sanger sequencing has been described by Sefrioui *et al*.[11] who showed that four of seven metastatic breast cancer patients studied had clear ESR1 mutations (two patients with Y537S mutations and two patients with D538G mutations).

Methods

Patients and samples

Between December 2011 and February 2018, we retrospectively collected 49 primary tumor samples from breast cancer patients before and after treatment by hormonal therapy at Dharmais Cancer Hospital-National Cancer Center. We included patients who were diagnosed with ER+, stage 3 and 4, and could undergo the hormonal therapy. Patients with early-stage cancers who had undergone chemotherapy were excluded from the study. Recurrences and progression were recorded during 7 years of follow-up time. The Institutional Ethical Review Board at Dharmais Cancer Hospital-National Cancer Center approved the study (number 025/KEPK/II/2018), and all patients provided informed consent.

Deoxyribonucleic acid extraction

Genomic deoxyribonucleic acid (DNA) was obtained from fresh-frozen primary breast tumor samples and extracted using the AllPrep DNA/RNA Mini Kit (QIAGEN) according to the manufacturer's protocol. The concentration and purity of DNA were measured using NanoDrop 2000 (Thermo Scientific). The purity was measured by calculating the absorbance 260 nm divided by the absorbance 280 nm (A260/280), with values ranging from 1.8 to 2.0.

Amplification of ESR1 gene

The target area of the ESR1 ligand-binding domain of 262 base pair was amplified using primers added by M13 sequence, which included forward primer 5'-CCCCTTCTAGGGATTTCAGC-3' and reverse primer 5'-AGTGGCTTTGGTCCGTCTC-3'. Polymerase chain reaction (PCR) was performed using PCR BigDye Direct Sanger Sequencing Kit (Applied Biosystem) containing DNA template (4 ng/µl) with a total reaction volume of 10 µl in a 0.2 ml microtube. As a positive control of D538G mutation, a ×100 dilution of gBlock synthetic gene fragment (Integrated DNA Technologies) containing the mutation was used.

The thermal cycler machine program for the PCR process was as follows: initial polymerization (one cycle) 95°C, 10 min → amplification process (35 cycles) 96°C, 3 s; 62°C, 15 s; 68°C, 30 s → post polymerization (one cycle) 72°C, 2 min. A total of 3 µl PCR products were run through electrophoresis using 2% agarose gel combined with ethidium bromide (EtBr) in tris-borate-EDTA (TBE) buffer at 100 Volt for 30 min. The length of DNA bands was measured through visualization using Gel Doc EZ Gel Documentation System (Bio-Rad).

Sequencing analysis

A total of 7 µl PCR product was added by 2 µl BigDye Direct Sequencing Master Mix (Applied Biosystem) and 1 µl BigDye M13 forward/reverse primer so that the total reaction became 10 µl. The sample was then carried out by a cycle sequencing process in the thermal cycler machine with the following program: Precycle (one cycle) 37°C, 15 min; 80°C, 2 min; 96°C, 1 min → cycle (25 cycles) 96°C, 10 s; 50°C, 5 s; 60°C, 75 s.

The sample was purified by adding 45 µl SAM solution and 10 µl XTerminator solution (Applied Biosystems) then vortexed at full speed for 30 min. After that, the sample was centrifuged at ×1000 g for 2 min. Sequencing was done in the Genetic Analyzer 3500 machine (Applied Biosystem) based on the Sanger sequencing principle. Analysis of exon 8 ESR1 gene sequencing was performed using Sequencing Analysis v5.4 software (Thermo Fisher Scientific).

Results

We retrospectively enrolled 107 patients from December 2011 to February 2018. Only 49 patients were included in the final analysis. Fifty-three patients who had undergone chemotherapy and five patients with ER-negative status were excluded

from this study [Figure 1]. Table 1 shows the characteristics of the 49 patients who received hormonal therapy. During the follow-up time, we found 79.6% of patients had disease progression and 14.3% of patients experienced recurrence. Of the 49 breast cancer patients who received hormonal treatment, 49% of patients passed away. Of the patients studied, 61.2% were stage 4. The molecular subtype of patients: 49.66% were in the luminal B category, 63.3% had low anatomic pathology levels, and 87.8% of the histopathology was ductal carcinoma. Of the 49 breast cancer patients who were treated hormonally, the average age was 46.14 ± 9.706 years (95% confidence interval mean = 43.35–48.93) [Table 1].

Table 1: Characteristic of 49 hormonal therapy patients with estrogen receptor-positive breast cancer

Patient's characteristics (n=49)	n (%)
Age (mean±SD)	46.14±9.606
Life information	
Alive	25 (51.0)
Dead	24 (49.0)
Stage	
IIIB	19 (38.8)
IV	30 (61.2)
PR status	
Negative	4 (8.2)
Positive	45 (81.8)
ER status	
Negative	0 (0)
Positive	49 (100.0)
HER-2 status	
Negative	40 (81.6)
Positive	9 (18.4)
KI-67	
Negative	3 (6.1)
Positive	46 (93.9)
Anatomical pathology grade	
Low	31 (65.5)
High	17 (34.7)
Molecular subtype	
Luminal A	22 (44.9)
Luminal B	27 (55.1)
Histopathology	
Ductal	43 (87.8)
Lobular	6 (12.2)
Progression	
Yes	39 (79.6)
No	10 (20.4)
Recurrence (20/49 pts)	
Yes	7 (35.0)
No	13 (65.0)

ER: Estrogen receptor, PR: Progesterone receptor, HER-2: Human epidermal growth factor receptor 2, SD: Standard deviation

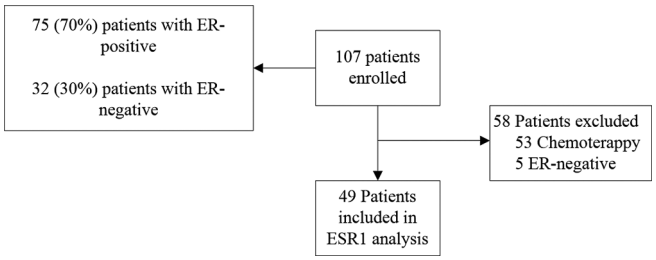

Figure 1: Patients' flowchart for ESR1 mutation analysis. ER: Estrogen receptor

Sanger sequencing was performed to detect the D538G mutation in 49 breast cancer patients. Results of sequencing data using Sequencing Analysis Software v5.4 showed no mutation observed on 49 samples of advanced breast cancer patients. As a control-positive D538G mutation, gBlock synthetic DNA showed base substitutions from A to G indicating the success of sequencing reaction [Figure 2].

Discussion

Over 70% of breast cancer is ER$^+$ at diagnosis.[17] Our study revealed the same findings; 75 of 107 (70.1%) advanced breast cancer patients had ER-positive status. This is reasonable considering that ER alpha (ESR1) is expressed in the majority of breast cancer and a major regulator of breast cancer development and progression.[18] Targeting the ER with hormonal therapies that suppress estrogen production was the first molecularly targeted treatment of breast cancer.[6,19] While many studies revealed that it has contributed significantly to reduce the development of the disease, recurrence, and mortality in breast cancer patients, we found 79.6% of patients with progression after receiving prior hormonal therapy in our study. Another study reported that approximately one-third of women treated with tamoxifen for 5 years have recurrent disease within 15 years.[20] Acquired hormonal therapy-resistant disease has thus been estimated to develop in up to one-quarter of all breast cancers.[21]

Preliminary functional studies indicate that some somatic mutations in ESR1 result in ER ligand-independent activity which is partially resistant to current hormonal therapy.[6,7,12,22,23] A study by Martin *et al*.[17] in breast cancer cell models showed

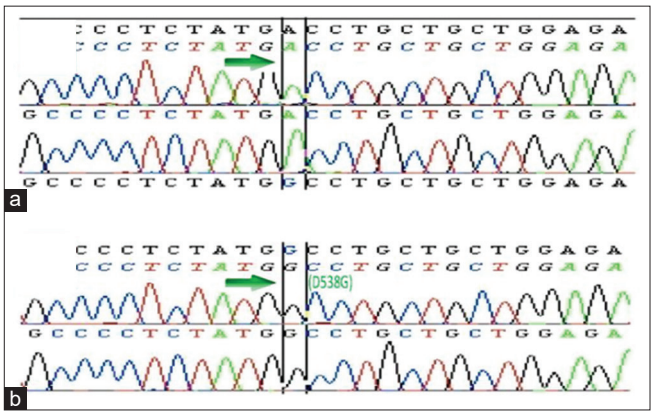

Figure 2: Visualization of electropherogram of ESR1 gene. (Source: Personal documentation). Representative traces of ESR1 mutation. (a) Wild Type exon 8 ESR1 gene, codon 538 (GAC, arrow). (b) gBlock synthesis deoxyribonucleic acid sequence. D538G mutation exon 8 ESR1, codon 538, shows aspartate acid → glutamic acid (GAC to GGC, arrow)

that the mutated ESR1 can control a cistrome similar to ligand-dependent wildtype ESR1 and associates with an altered protein interactome enabling ligand-independent proliferation. Among these mutations, the most common are Y537S, Y537N, Y537C, and D538G, which cover >80% of ESR1 mutations and are clustered in the ligand-binding domain.[24] In Indonesia, the genetic testing for ESR1 mutation is not yet commonly available due to limited funding, limited sophisticated detection technique, and lack of information about ESR1 mutation profiling in our population. To reveal the frequency of ESR1 mutation, we performed Sanger sequencing as a preliminary study for our institution. This is our first study to investigate the exon 8 ESR1 gene mutation in primary tumor samples from advanced breast cancer patients. Based on the results, none of the 49 breast cancer patients harbored any ESR1 mutations in exon 8, a result which has several implications. First, in terms of sample type that we used, we have learned that the absence of detectable mutations in the primary breast tumors suggests clonal evolution as the mechanism of resistance.[7] These mutations emerge either through a clonal selection of very low abundance resistance clones or are acquired later in the disease course under the selective pressure of hormonal therapy.[10] Thus, ESR1 mutations are characteristically absent in primary tumors and are an unlikely mechanism of primary resistance.[25,26] Most patients with tumors harboring ESR1 mutation seem to experience an extended clinical course before detection of mutation, supporting the idea that this is largely a secondary (acquired) resistance mechanism that emerges after long-term hormonal therapy.[27] This is consistent with previous studies that revealed low or undetectable rates of ESR1 mutations in primary breast cancers using Sanger sequencing.[9,11,24] Large-scale genomic efforts such as The Cancer Genome Atlas project for 962 breast cancer samples indicated that ESR1 mutations were present in only 0.5% of primary breast tumor cases.[25]

Second, in terms of the technique that we used, the lack of ESR1 mutation is more likely due to the low frequency of ESR1 mutation in our samples, whereas the sensitivity of Sanger sequencing can only detect mutations with a frequency above 10%.[28] However, we could not confirm whether is there any ESR1 mutation or not in our samples, unless the results can be compared with other advanced techniques. One of the methods developed in recent years is droplet digital PCR (ddPCR) technology. Several studies found that the ESR1 mutation rate in primary breast cancer is higher than previously estimated.[8] ddPCR can be used for detection of the mutation with high sensitivity, up to 0.25 copies/μL.[29] According to other studies with various cutoff values for detection, hotspot mutations of ESR1 can be found in approximately 2.5%–7% of primary breast cancer.[24]

Even though Sanger sequencing method has failed to detect ESR1 mutation from primary breast tumor due to its sensitivity, a study from Sefrioui *et al*.[11] suggests that Sanger sequencing is still capable to detect the ESR1 mutation in metastatic cancer, due to clonal evolution as the mechanism of resistance. Another source showed that Sanger sequencing is feasible to detect ESR1 mutation in single circulating tumor cell DNA samples.[30] That evidence creates a research opportunity for us to expand the ESR1 mutation study in the future with other sample types. Finally, the small number of patients and the lack of metastatic or circulating DNA samples, which could be used to compare the existence of ESR1 mutations in primary and metastatic sites, represent the limitations of this study. Moreover, the limited advanced detection technique remains an obstacle for our institution.

Conclusion

Sanger sequencing failed to detect ESR1 mutation in primary breast tumor samples in our study. Hence, other advanced molecular techniques such as ddPCR or NGS should be performed and compared with Sanger sequencing results.

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Conflicts of interest

There are no conflicts of interest.

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