RESEARCH ARTICLE

Screening of *BRCA1/2* Mutations Using Direct Sequencing in Indonesian Familial Breast Cancer Cases

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Abstract

Breast cancer has emerged as the most prevalent cancer among women worldwide, including in Indonesia. The contribution of genes associated with high-risk breast-ovarian cancers, *BRCA1* and *BRCA2*, in the Indonesian population is relatively unknown. We have characterized family history of patients with moderate- to high-risk of breast cancer predisposition in 26 unrelated cases from Indonesia for *BRCA1/2* mutation analyses using direct sequencing. Known deleterious mutations were not found in either *BRCA1* or *BRCA2* genes. Seven variants in *BRCA2* were documented in 10 of 26 patients (38%). All variants were categorized as unclassified (VUSs). Two synonymous variants, c.3623A>G and c.4035T>C, were found in 5 patients. One variant, c4600T>C, was found in a 38 year old woman with a family history of breast cancer. We have found 4 novel variants in *BRCA2* gene including c.6718C>G, c.3281A>G, c.10176C>G, and c4490T>C in 4 unrelated patients, all of them having a positive family history of breast cancer. In accordance to other studies in Asian population, our study showed more frequent variants in *BRCA2* compared to *BRCA1*. Further studies involving larger numbers of hereditary breast cancer patients are required to reveal contribution of *BRCA1/2* mutations and/or other predisposing genes among familial breast cancer patients in Indonesia.

Keywords: Familial breast cancer - *BRCA1/2* - Indonesian population - germline mutation

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Introduction

Breast cancer is the most common cancer diagnosed among women worldwide including in Indonesia and the incidence is continuously increasing. (Akhsan and Aryandono, 2010; Jemal et al., 2011; Siegel et al., 2014) Although the mortality rate due to breast cancer improves during the past decades, social and economical burdens to the family and health system remain as the major problem in Indonesia. Especially in family with positive history of breast-ovarian cancer, other family members often feel worry for the higher risk to develop breast cancer (Purnomosari et al., 2007; Akhsan and Aryandono, 2010; Jemal et al. 2011).

Majority of breast cancer cases are sporadic without any familial predisposition. Around 5%-10% are diagnosed as hereditary cancer due to mutations in a single dominantly acting gene. However, only 25%-50% of hereditary breast cancers can be explained by mutations in the two most important breast cancer genes, *BRCA1* and *BRCA2* (Kobayashi et al. 2013). Hereditary breast cancer types are suggested in individuals with early onset of breast cancer (less than 40 years old), multiple primary cancers in a patient, clustering of breast-ovarian cancer within family, bilateral breast cancer, and first degree relatives of mutation carriers. Familial breast cancers have distinct morphology, response to treatment, and clinical outcome. *BRCA1*-associated breast cancers are predominantly triple-negative, basal-like type, and high grade. Family history and pedigree construction remain the most common approach to initially recognize hereditary/familial breast cancer. Recently, relying on family history is relatively difficult because of small size of modern family and indecisive family history records (Daly et al., 2010; Gage et al., 2012).

Germline mutations in *BRCA1* (MIM#113705; 17q chromosome) and *BRCA2* (MIM#600185; 13q chromosome) genes are responsible for 25-50% of hereditary breast cancer. Women with *BRCA1/2* mutations have an increased risk for breast cancer, ovarian cancer, fallopian tube cancer and other types of cancer. The risk for *BRCA1*-mutation carriers to develop breast cancer and ovarian cancer by age of 70 is 65-80% and 37-62%, respectively. While women with *BRCA2* mutations have 45-85% and 11-23% lifetime risks to develop breast and ovarian cancer, respectively. The penetrance depends significantly on type of mutations as well as exogenous factors (Janavicius et al., 2010 ; Narod, 2010). In total

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1,781 distinct variants in *BRCA1* gene and 2,000 in *BRCA2* gene have been documented in the Breast Cancer Information Core (BIC) database (January 2016). (*The Breast Cancer Information Core Database., 2012.*) *BRCA1/2* proteins function as tumor suppressor and play an important role in the maintenance of genomic stability, DNA damage repair, cell cycle checkpoint, and transcriptional regulation (Roy et al., 2011; Kim et al., 2012).

In developed countries, genetic counseling and genetic testing for *BRCA1* and *BRCA2* are included in the standard of care for women at high-risk for breast-ovarian cancers. Genetic testing for *BRCA1/2* is not yet commonly available in developing countries including in Indonesia due to limited health funding and limited information about prevalence of mutations of these genes (Kang et al., 2013; Kobayashi et al., 2013). Until now, only one study has been addressed to detect mutations in *BRCA1/2* genes in Indonesia population. To reveal prevalence of BRCA mutations in our population, we performed direct sequencing in 26 unrelated Indonesian patients with high risk of breast cancer predisposition for *BRCA1/2* mutation analysis.

Materials and Methods

Ethics clearance

All patients have provided written inform consent to participate in this study and permit that the DNA samples would be genetically analyzed. This study has been approved by the ethical committees of The National Cancer Center of Dharmais Hospital Jakarta, Indonesia.

Patients and selection criteria

A total of 26 unrelated breast cancer patients were recruited from The National Cancer Center of Dharmais Hospital Jakarta, Indonesia. Breast cancer patients with moderate to high risk of a hereditary predisposition were recruited with the following criteria: (1) early-onset breast cancer (≤40 years), with or without a family history of breast or ovarian cancer in first- or second-degree relative; (2) younger than 50 years of age at diagnosis of breast cancer together with at least one first- or second-degree relative having breast or ovarian cancer at any age; or (3) bilateral breast cancer; or (4) two or more first- or second-degree relatives with breast and/or ovarian cancer, regardless of age. The clinicopathological characteristics of 26 breast cancer patients recruited in this study were summarized in Table 1. The eligible patients were then proposed for genetic counseling prior to consent. Collections of blood and tumor tissue samples were performed after inform consent from the selected patients. Patients with positive BRCA mutation testing were further informed for possible clinical surveillance and were offered to recruit their first-degree relatives for BRCA genetic testing.

DNA extraction

BRCA1 and *BRCA2* mutation analysis was performed using genomic DNA extracted from 150 μl peripheral blood sample (for each patient) with DNA isolation kit (Roche, Germany). A ratio of absorbance at 260 nm and 280 nm of the purified DNAs between 1.7 - 1.8 is generally accepted for PCR and subsequent direct sequencing. Agarose gel electrophoresis was used to check the DNA integrity.

Preparation for PCR

For DNA amplification, primers that have been previously used by De Leeneer et al. (2012) and human genome variation society (HGVS) were used in this study. A total of 39 and 52 PCR reactions were performed for amplification of *BRCA1* and *BRCA2* genes, respectively. Polymerase chain reaction was conducted in 25 μl PCR cocktail containing: 62.5 ng genomic DNA, 1X PCR buffer MgCl2 (Roche, Germany), 0.2 mM dNTP mx (Roche, Germany), 10 pmol of each primer (FirstBase, Singapore), 1.25 IU of Taq DNA polymerase (Roche, Germany). All reactions were performed in a thermal cycler (MyCycler, Biorad) with a program: pre-denaturation (95^{o}C in 5 min); denaturation, annealing, and elongation in 40 cycles (95^{o}C in 20 sec, 59-66^{o}C in 30 sec, 72^{o}C in 40 sec, respectively); and final elongation (72^{o}C in 5 min). Polymerase chain reaction was not conducted at the exon 9 of *BRCA1* because we could not obtain optimal temperature for the annealing stage. In addition, exon 21 and 26 of *BRCA2* were also not also amplified because the regions were not eligible for PCR amplification (short exon). PCR products were checked in agarose gel DNA electrophoresis with 1X Tris-Acetate-EDTA buffer system (2.5% agarose gel) prior to sequencing.

Direct sequencing of PCR products

PCR products were then purified using high pure PCR product purification kit (Roche, Germany). Precipitation of purified PCR products was carried out using DYEnamic ET terminator kit manual (GE Healthcare, USA). Direct sequencing was performed in GE MegaBACE 750 DNA analyzer/capillary electrophoresis (GE Healthcare, USA) following the manufacturer's instructions. DNA sequences were then analyzed using BioEdit Alignment software (Carlsbad, USA) and reviewed manually by two independent persons.

Mutation database

The genetic variants identified in our patients were compared with online published database from the Breast Information Core Database of National Institute of Health (BIC). Any findings of *BRCA1/2* mutations were documented and compared.

Family history and clinicopathological characteristics

Detailed pedigree assessment and construction were formulated from deep anamnesis from each patient. A standard epidemiological questionnaire, including a detailed personal and family history, was also collected. Epidemiological questionnaire provided some information related to age at breast cancer diagnosis; marital status; age of menarche; other cancer diagnosed in the patient; a family history of breast, ovarian, and other cancers in first- or second-degree relatives; history of breast or ovarian surgery. Patient's medical records were collected and

reviewed to complement the personal and familial history, clinicopathological data, and follow-up of treatment. Tumor stage was determined based on the seven edition of the American Joint Committee on Cancer staging system. Expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 from immune-staining of the tumor tissues were documented to sub-classify patients.

Results

Patient characteristics

A total of 26 patients with moderate and high risk of a hereditary breast cancer predisposition with a mean age of 44.8 years old (range 29-58 years old) were recruited to this study. All of them were referred and treated at the National Cancer Center of Dharmais Hospital, Jakarta,-Indonesia. All patients had a family history of breast cancer. No patient was identified with a family history of ovarian cancer in the first and second degree family. Histological data showed that 76.9% patients had invasive ductal carcinoma. In our study, 69.2% patients were positive for estrogen-receptor and 7.6% were positive for progesterone-receptor. The clinicopathological data of these patients are summarized in Table 1.

Table 1. The clinicpathological characteristics of patients in this study

Characteristics	Number
Total patients (N)	26
Age at diagnosis (years)	
20-29	1
30-39	5
>40	20
Family history	
Breast only	26
Both breast and/or ovarian	-
Bilateral breast cancer	-
Tumor histology	
IDC	20
DCIS	1
Medular	1
Mucinous	1
NA	3
Stage	
I	-
II	10
III	7
IV	-
N/A	9
Estrogen receptor	
Positive	18
Negative	2
NA	6
Progesterone receptor	
Positive	2
Negative	19
NA	5
HER2	
Positive	3
Negative	18
NA	5

IDC: invasive ductal carcinoma; DCIS: ductal carcinoma in situ; NA: not available

BRCA1/2 sequence variants

Direct sequence analysis of all coding exons in *BRCA1* and *BRCA2* were performed in 26 familial breast cancer patients. Seven variants at the *BRCA2* gene were identified. Deleterious *BRCA1/2* gene mutations were not documented. In total, 10 (10/26, 38.5%) breast cancer patients had single nucleotide substitution in *BRCA2* gene. Of these 10 patients, 5 patients (5/26, 19.2%) had synonymous mutation and 5 other patients (5/26, 19.2%) showed missense mutation (Table 2). All variants documented in our study were classified as variants of uncertain significant (VUSs). According to the BIC, four out of the 10 VUSs in *BRCA2* gene were novel substitutions which had not yet been published before. *BRCA1* mutations or variants were not found in our study.

A variant found in *BRCA2* exon 11 c.6718C>G led to amino acid glutamine to glutamic acid substitution. While alteration of *BRCA2* c.3281A>G caused substitution of lysine to arginine. *BRCA2* variant at c.4490T>C resulted in substitution of phenylalanine to serine. A single nucleotide substitution was identified at the exon 27 c.1017C>G and caused conversion of amino acid glutamine to glutamic acid. All 4 variants were novel mutations and were not registered before at the BIC (Table 2).

The c.3623 A > G synonymous mutation was found in 4 unrelated breast cancer patients with diagnosis at the age of 34 years old, 37 years old, 42 years old, and 45 years old. All of these patients had first or second degree familial history of breast cancer in which her maternal aunt, mother, mother, mother and maternal cousin were respectively affected. In addition, 4 variants including c.4035 T > C (synonymous), c.6718 C > G (missense), c.3281 A > G (missense), c.4600 T > C (missense) were identified from respectively 34 years old, 46 years old, 52 years old, 38 years old women whose their mothers had

Table 2. Sequence variants identified in the *BRCA2* gene

Genetic variants	Location	AA change	Type of mutation	Clinical relevance	Reported/ novel by BIC	Frequency, N=26
c.3623A>G	Exon 11	p.K1132K	Synonymous	VUS	Reported	4/26 (15,4%)
c.4035T>C	Exon 11	p.V1269V	Synonymous	VUS	Reported	1/26 (3,8%)
c.6718C>G	Exon 11	p.Q2164E	Missense	VUS	Novel	1/26 (3,8%)
c.3281A>G	Exon 11	p.K1018R	Missense	VUS	Novel	1/26 (3,8%)
c.10176C>G	Exon 27	p.E3316Q	Missense	VUS	Novel	1/26 (3,8%)
c.4490T>C	Exon 11	p.F1421S	Missense	VUS	Novel	1/26 (3,8%)
c.4600T>C	Exon 11	p.H1458Y	Missense	VUS	Reported	1/26 (3,8%)

AA: amino acid; BIC: Breast Cancer Information Core; VUS: variant of unknown significance

Table 3. Sequence variants identified in the *BRCA2* Gene and Clinical Characteristics

Genetic variants	Tumor	ER/PR/HER2	Age	Family history of breast cancer
c.3623A>G	IDC	-/-/-	37	mother
	IDC	-/-/2+	34	maternal aunt
	IDC	-/-/-	42	mother
	IDC	-/-/-	45	mother, maternal cousin
c.4035T>C	IDC	-/-/-	34	mother
c.6718C>G	Mucinous	-/-/-	46	mother
c.3281A>G	DCIS	-/-/-	52	mother
c.1017C>G	IDC with DCIS	-/-/-	46	cousin
c.4490T>C	IDC	-/-/-	46	maternal aunt
c.4600T>C	IDC	-/-/-	38	mother

DC: invasive ductal carcinoma; DCIS: ductal carcinoma in situ; NA: not available; ER: estrogen receptor; PR: progesterone receptor; Age – age at diagnosis of breast cancer

breast cancer. Two variants, c.1017 C > G and c.4490 T > C missense mutations, were recognized in two unrelated women both at the age 46 years old with family history of breast cancer in her cousin and maternal aunt, respectively.

In our study, the histopathological features of breast cancer with high risk of hereditary predisposition were dominated by invasive ductal carcinoma (IDC) with negative immunohistochemical staining for ER, PR, and HER2 (triple negative).

Discussion

Segregation analysis studies have shown that approximately 5,-10% breast and/or ovarian cancer cases are inherited with autosomal dominant pattern. Germline mutations of BRAC1 and *BRCA2* genes account for the genetic predisposition and significantly increase the risk of breast and ovarian cancer. Individuals with *BRCA1/2* mutations have probability 45-55% to develop breast cancer and 17-39% to develop ovarian cancer at the age of 70 years. (Chen et al., 2007; Ripperger et al., 2009) *BRCA1/2* mutations are more frequent among women with first and second degree history of breast or ovarian cancer, a personal history of young age breast cancer, triple-negative subtype breast cancer (Chen et al., 2007; Daly et al., 2010; Hartman et al., 2012). The frequency of *BRCA1/2* mutations varies greatly among different ethnic groups and countries (Hall et al., 2009). In developed nations, genetic cancer risk assessment guideline (including genetic testing) for inherited breast cancer susceptibility have become a standard for clinical management for high-risk patients (Narod et al., 2010). In developing countries including Indonesia, management of patients with high-risk for a predisposition of familial breast and ovarian cancer are not yet standardized. Study focusing on familial breast cancer and BRCA mutation spectrum in developing countries is often limited (Agarwal et al., 2009; Kim et al., 2013).

In our study, from a total of 26 breast cancer patients with hereditary predisposition, we did not find any deleterious *BRCA1/2* mutation. Although deleteriuos mutations in *BRCA1/2* genes were not found, we have documented variants at the *BRCA2* gene in 10 patients. Five patients (19.2%) harbored synonymous mutation and the other 5 patients (19.2%) carried missense mutations. In the synonymous mutations, sequence alternations were identified without any amino acid substitution. Two synonymous mutations in the *BRCA2*, c.3623A>G and c.4035T>C, were detected in 4 and 1 breast cancer patients, respectively. All of them have family history of breast cancer and manifest as infiltrative ductal carcinomas.

Missense mutations, on the other hand, led to a single amino acid change. Both synonymous and missense mutations identified in our study were classified as variants of unknown significance (VUSs). We reported 4 novel *BRCA2* variants including three variants were located at the exon: 11 c.6718C>G (p.Q2164E), c.3281A>G (p.K1018R), and c.4490T>C (p.F1421S) as well as a variant at the exon 27 c.10176C>G (p.Q3316E). To predict possible functional impact for the novel *BRCA2* variants, we used in silico analysis using Align-GVGD and PANTHER (Brunham et al., 2005; Tavtigian et al., 2006). Align-GVDV calculates Grantham Variation (GV) and Grantham Deviation (GD). GV shows degree of biochemical variation among amino acid at a given position within multiple sequence alignments (MSA). GD predicts the distance between a missense substitution and range of variation observed at its position in the alignment. PANTHER calculates subPSEC scores that have been previously shown to be able to statistically differentiate Mendelian disease-associated missense mutations from random coding polymorphisms. The missense variant Q2164E has subPSEC score -2.07, Pdeleterious 0.28, GV=353.8, and GD=0. The second variant pK1018R has subPSEC score -1.38, Pdeleterious 0.16, GV=266, and GD=0. The pE3316Q variant has subPSEC score -1.9, Pdeleterious 0.25, GV=112, and GD=2.03 and the pF1421S variant has subPSEC score -1.8, Pdeleterious 0.24, GV=112, and GD=0. GV=0 represents in variant residue in the alignment, GV=60-65 indicates upper limit of conservative variation across species, and GV > 100 designates residue with slight functional constraint. GD=0 represents a missense variant within cross-species range of variation, GD=60-65 corresponds with upper limit of a conservative missense variant, and GD > 100 indicates radical substitution (Tavtigian et al. 2008). Using PANTHER, subPSEC scores have value ranging from 0 (neutral) to 10 (most likely as deleterious mutation). SubPSEC is able to be converted into Pdeleterious value as equation 1 as the most probable for detelerious effect (Brunham et al. 2005). Therefore, our novel identified

VUSs are not likely to affect *BRCA2* protein functions.

Only one study has previously addressed to *BRCA1/2* germline mutations in familial breast cancers among Indonesian population. This study focused on young-onset (<41 years-old) breast cancer patients using PCR-DGGE as pre-screening mutation analysis. Thirty three mutations at *BRCA1/2* mostly VUSs were found among 120 early-onset Indonesian breast cancer patients (Purnomosari et al., 2007). Therefore, the contribution of *BRCA1/2* gene mutations in familial breast cancer cases among Indonesian population is relatively uncovered. Among Asian population, most studies showed more frequent *BRCA2* than *BRCA1* mutations in familial breast-ovarian cancer patients (Kim et al., 2013; Han et al., 2013). In multiethnic groups such as in Indonesia, prevalence of *BRCA1/2* mutations is relatively difficult to be estimated. In addition, other genes or genetic modifiers might contribute to the familial breast cancer cases in our population (Bruin et al., 2012). Further studies with larger cohort addressing genomic *BRCA1/2* rearrangement and mutation screening of gene functionally related to *BRCA1/2* including PALB2, NBS1, CHEK2, ATM, and BRIP1 are warranted (Seong et al., 2009; Kang et al., 2010; Zhang et al., 2011; He et al., 2012).

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