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Introduction

Globally, breast cancer (BC) ranks highest and is the most prevalent carcinoma among women in Indonesia, accounting for 48,998 new cases (30.5%) with 5 years prevalence of 171.005 (41.7%) (Ferlay, 2013). Early onset BC occurs in patients aged 40 years or younger and its incidence varies among races (Anders et al., 2009). Early onset of BC tends to occur with hormone receptor negativity in larger and more aggressive, high grade tumors with hormone receptor negativity, and HER2 overexpression (Assi et al., 2013), with greater risk of contralateral breast cancer in 15 years (Metcalfe et al., 2011).

In the United States, only 6.8% of BC patients are below 40 years (Anders et al., 2009), whereas in Asia its incidence rate could reach 9.5-11% (Han et al., 2004). Early onset cases in Dr. Sardjito Hospital Yogyakarta between the years 2012-2015 showed incidence rates ranging from 12.8 – 19.7% (unpublished data).

One of the major risk factors for early onset BC is a history of BC in the family, which involves inherited genetic mutations, in particular the major susceptibility genes BRCA1 and BRCA2. However, BRCA1 and BRCA2 have been shown to account for up to only 15% of familial early onset BC cases (Stratton and Rahman, 2008). One study in China showed that among early-onset BC without a family history of ovarian or BC, these genes were responsible for about 3% and 0%, respectively (Chen et al., 2009). The same type of study in Indonesia showed that BRCA1 and BRCA2 were responsible for 8.3% of the cases (Purnomosari et al., 2007), accounts for only a small percentage of early-onset non-familial BC, indicating other low penetrance susceptibility genes could exist and play a role in tumorigenesis.

Genetic mutations occur in about 1% of the population and the most common type of germline variations are single nucleotide polymorphisms (SNPs) (Taylor et al.,...
Materials and Methods

Study population and sample collection

This research was a hospital-based case control study that included 98 early-onset BC cases (<40 years old at diagnosis) and 101 late-onset cases (>55 years old) as the controls. These cases were pathologically diagnosed and collected between the year 2006–2013 at the Dr. Sardjito Hospital Yogyakarta, Indonesia. The study was approved by the Medical and Health Research Ethics Committee of Faculty of Medicine Universitas Gadjah Mada (KE/FK/779/EC and KE/FK/570/EC/2015).

DNA extraction and genotyping

Spectrophotometric measurement of the absorbance ratio 260/280 nm were used to determine DNA purity and concentration. Genotyping of the 6 genes was performed using Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) analysis. Details of selected SNPs, primer sequences used for PCR, sizes of PCR products and restriction endonuclease, (New England Biolabs, Ipswich, Massachusetts, United States) used for digestion are described (Table 1). Restriction digested fragments from all of the SNPs were analyzed with 3% agarose gel electrophoresis (except for FGFR2, 4% of agarose).

Statistical analyses

Data analysis used SPSS-21 software (IBM Corp., Chicago). The Hardy-Weinberg equilibrium was first applied to both case and control groups, separately for each variant before association analyses. Significance level (α) used was 5%. To calculate odds ratios (ORs), unconditional logistic regression model was used with 95% confidence interval (CI) as estimates of relative risk (RR) for the single locus alleles and genotypes.

Results

In this hospital-based case and control study, we analyzed 6 genes’ polymorphisms in 98 early-onset BC patients and 101 late-onset BC patients as the controls. The mean age at diagnosis for early-onset group was 36±4.2 years, while the late-onset group was 62±6.9 years. The Hardy-Weinberg equilibrium analysis showed no significant deviations between early and late-onset BC patients (p>0.05; data not shown).

Genotype distribution of each evaluated gene can be found in Table 2. Four genes showed high frequency in heterozygote genotypes, i.e. Arg/Pro of p53, T/G of MDM2, A/C of p21 and C/T of FGFR2 with proportion of 48%, 55.1%, 45.9% and 45.9%, respectively. There was no different proportion between early and late onset cases. On the other hand, genotype frequencies of HER2 and ER1 were different from the other 4 genes, as the most frequent genotype for HER2 was the homozygote wildtype (Ile/Ile), while for ER1 it was the homozygote mutant (G/G).

### Table 1. Primers and Restriction Enzyme Used for Genotyping 6 Genes

| Gene | SNP | rs number | Primer F | Primer R | PCR product | Enzyme |
|------|-----|-----------|----------|----------|-------------|--------|
| p53  | Pro72Arg | rs1042522 | 5'- TTG CCG TCC CAA GCA ATG GAT GA -3' | 5'- TCT GGG AAG GGA CAG AAG ATG A -3' | 199bp | BstU1 |
| MDM2 | 309 T>G | rs2279744 | 5'- GAT TTC GGA CGG CTC TCG CGG C-3' | 5'- CAT CCG CAC CTC CCG GC C-3' | 121bp | PstI |
| HER2 | Ile655Val | rs1136201 | 5'- AGA GAG CCA GCC TCT GCA GTG CCA T-3' | 5'- TCC GTT TCC TGC AGT CTC CCG A-3' | 148bp | BsmAI |
| ER1  | 594 A>G | rs2228480 | 5'- GAG GAG ACG GAC CAC AGC CAC -3' | 5'- GCC ATT GGT GTG GGA TGC ATG C-3' | 227bp | BglI |
| p21  | Ser31Arg | rs1801270 | 5'- ATA GTG TCT AAT CTC CG C CG -3' | 5'- AAG TCA CCC TCC AGT GTG GT -3' | 245bp | BglI |
| FGFR2 | - | rs2981582 | 5'- CCC TTT GGA GAC AAC GTG AGC C -3' | 5'- CAG GCA CCA GGT GGA CTC TGC-3' | 176bp | HinP11 |
Gene Polymorphisms and Early Onset Breast Cancer

Similar to the previous studies, in the current study, Ser31Arg polymorphisms were found to be associated with the increased risk of early onset BC with OR of 2.40 (95% CI: 1.13–5.67) and 2.54 (95% CI: 0.31–16.51), respectively, suggesting that Ser31Arg polymorphisms are correlated with the increased risk of early onset BC. Similarly, T/T genotype and T allele of FGFR2 rs2981582 were found to increase the risk of early onset BC with OR of 2.40 (95% CI: 1.08–5.32) and 1.56 (95% CI: 0.21–3.84) respectively.

Discussion

Poor prognosis and low survival are linked to early onset of BC and accordingly, preventive measures by recognizing the risk factors are very much needed. Genetic variations in genes that regulate crucial processes such as cell apoptosis, proliferation and DNA repair are potential candidates for breast cancer risk factors. Recent research that was conducted in many different countries shows different outcomes among different populations.

In this study, we evaluated 6 genes that have been linked with susceptibility to early onset of BC, only two of which were significantly associated with the risk of early onset BC. The frequency of Ser31Arg polymorphisms was higher in early onset cases compared to late onset cases, while the frequency of T/T genotype of FGFR2 rs2981582 was higher in late onset cases compared to early onset cases.

Table 2. Genotype and Allelic Frequencies of P53 Pro72Arg, MDM2 SNP309, P21 Ser31Arg, ER SNP594, HER2 Ile655Val, and FGFR2 rs2981582 in Breast Cancer Patients with Different Age of Onset

| Polymorphisms       | Genotype/Allele | Cases (<40) n=98 | Controls (>=55) n=101 | p-value | OR   | 95% CI     |
|---------------------|-----------------|-----------------|-----------------------|---------|------|------------|
| HER2 Ile133Val      | I/I             | 73 (74.5%)      | 74 (73.3%)            | 0.614   | ref  | ref       |
|                     | I/V             | 21 (21.4%)      | 25 (24.8%)            | 0.852   | 0.42-1.74 |
|                     | V/V             | 4 (4.1%)        | 2 (2.0%)              | 2.027   | 0.31-16.51 |
|                     | I               | 167 (85.2%)     | 173 (85.6%)           | 0.901   | 1.036 | 0.57-1.87 |
|                     | V               | 29 (14.8%)      | 29 (14.3%)            |         |      |            |
| ER1 594A>G          | A/A             | 5 (5.1%)        | 5 (5%)                | 0.52    | ref  | ref       |
|                     | A/G             | 35 (35.7%)      | 44 (43.6%)            | 0.713   | 0.38-1.33 |
|                     | G/G             | 58 (59.2%)      | 52 (51.5%)            | 0.897   | 0.21-3.84 |
|                     | A               | 45 (23%)        | 54 (26.7%)            | 0.384   | 0.817 | 0.51-1.32 |
|                     | G               | 151 (77%)       | 148 (73.3%)           |         |      |            |
| p53 Arg72Pro        | A/A             | 33 (33.7%)      | 25 (24.8%)            | 0.183   | ref  | ref       |
|                     | A/P             | 47 (48%)        | 50 (49.5%)            | 0.637   | 0.31-1.29 |
|                     | P/P             | 18 (18.4%)      | 26 (25.7%)            | 0.489   | 0.20-1.17 |
|                     | A               | 114 (58.2%)     | 99 (49.0%)            | 0.067   | 0.691 | 0.46-1.05 |
|                     | P               | 82 (41.8%)      | 103 (51.0%)           |         |      |            |
| MDM2 SNP309         | T/T             | 20 (20.5%)      | 23 (22.8%)            | 0.644   | ref  | ref       |
|                     | T/G             | 54 (55.1%)      | 49 (48.5%)            | 0.789   | 0.39-1.61 |
|                     | G/G             | 24 (24.5%)      | 29 (28.7%)            | 1.051   | 0.47-2.36 |
|                     | T               | 94 (48.0%)      | 95 (47.0%)            | 0.853   | 1.038 | 0.7-1.54  |
|                     | G               | 102 (52.0%)     | 107 (53.0%)           |         |      |            |
| p21 Ser31Arg        | C/C             | 21 (21.4%)      | 30 (29.7%)            | 0.047   | ref  | ref       |
|                     | C/A             | 45 (45.9%)      | 53 (52.5%)            | 1.213   | 0.61-2.41 |
|                     | A/A             | 32 (32.7%)      | 18 (17.8%)            | 2.54    | 1.14-5.67 |
|                     | C               | 87 (44.4%)      | 113 (56%)             | 0.021*  | 1.591 | 1.07-2.36 |
|                     | A               | 109 (55.6%)     | 89 (44%)              |         |      |            |
| FGFR2 rs2981582     | C/C             | 26 (26.5%)      | 37 (36.6%)            | 0.091   | ref  | ref       |
|                     | C/T             | 45 (45.9%)      | 48 (47.5%)            | 1.334   | 0.70-2.55 |
|                     | T/T             | 27 (27.5%)      | 16 (15.8%)            | 2.401   | 1.08-5.32 |
|                     | C               | 97 (49.5%)      | 122 (60.4%)           | 0.028*  | 1.556 | 1.05-2.32 |
|                     | T               | 99 (50.5%)      | 80 (39.6%)            |         |      |            |

*, p<0.05; OR, odds ratio; CI, confidence interval
P21 Se31Arg polymorphism

Results of the present study showed that AA genotype and A allele of P21 were significantly more frequently found in early onset cancer cases and had a 2.540 increased risk of early-onset BC (95% CI: 1.138-5.668). P21 (also identified as CDKN1A), which encodes p21/WAF1/CIP1, a cyclin-dependent kinase inhibitor, is considered to significantly influence cell cycle control (Gartel and Radhakrishnan, 2005). One polymorphism of P21 that has received much attention is Ser31Arg (rs1801270), which substitutes A for C in the third base of codon 31 of P21 results in a serine to arginine amino acid substitution in the DNA-binding zinc finger motif of the protein (Mousses et al., 1995). Thus, it is very likely this polymorphism is also involved in cancer development (Lukas et al., 1997).

Several results support the role of P21 Ser31Arg polymorphism in BC risk (Keshava et al., 2002; Ma et al., 2006), but the results are inconclusive, possibly due to small effect of the polymorphism on BC risk and the relatively small number of samples used in the studies.

There have been several studies of P21 Ser31Arg polymorphism that were done to determine the risk of BC incidence. One meta-analysis study by Qiu and colleagues (Qiu et al., 2010) reported that the genotype C/C significantly increased the risk of breast cancer by 1.496 times (95% CI: 1.164-1.924) for Caucasians. However, for the Asian population, there was no statistically significant increase in risk (OR 0.896; 95% CI:0.424-1.893).

FGFR2 rs2981582 polymorphism

FGFR2 is a member of the FGFR family that contributes to cell growth, malignancy, motility, and angiogenesis. Various meta-analysis studies have been conducted and reported that SNP FGFR2 rs2981582 TT is linked to an increased risk of BC, but until now the exact mechanism of carcinogenesis linking SNP FGFR2 rs2981582 TT to BC, especially early breast cancer onset has not been found (Zhang et al., 2010; Peng et al., 2011).

In this study, frequency of C/C genotype was higher in patient with late onset compared to early onset (36.6% vs 26.5%) while T/T genotype was higher in younger cases compared to the older patients (27.5% vs 16.8%), but the result was not statistically significant. Interestingly, proportion of allele T was significantly different in early onset cases.

SNP FGFR2 rs2981582 intron 2 alters the binding of two transcription factors, Oct-1 / Runx2 and C / EBpβ, resulting in increased expression of the FGFR2 gene in cell lines and mammary tissue (Jia et al., 2010). This overexpression is confirmed by the study of Rebbeck (Rebbeck et al., 2009) that reported FGFR2 over expression occurs in 10-15% of breast tumors, but FGFR2 expression is lower in normal breast tissue in someone who has T allele (Sun et al., 2010). In addition, the in vitro evidence also shows that the mechanism of carcinogenesis of the SNP is more directed toward the anti-apoptosis effect than the mitogenic effects which are partly mediated through the down-regulation of FOXO protein synthesis. Amplification of the FGFR2 gene due to SNP results in an increase in intracellular screening which affects the decrease of FOXO protein. FOXO proteins are involved in apoptotic induction, DNA damage repair, ROS detoxification, and down-regulation of ER-alpha and ER-beta transcription activities. Therefore, decreased protein synthesis of FOXO will decrease apoptosis, ROS detoxification function, DNA repair, and down-regulation of ER transcription activity (Murillo-Zamora et al., 2013).

All of the above findings significantly support our results that show FGFR2 gene polymorphism rs2981582 TT can be linked to increased risk of early onset BC in Yogyakarta, Indonesia. This finding is consistent with a study conducted by Fu et al., (2012) which stated that TT and T allele genotypes in FGFR2 polymorphism rs2981582 increase the risk of early-onset BC in South Chinese Han populations with ORs of 3.062 (95% CI: 1.229-7.629) and 1.606 (95% CI: 1.084-2.380), respectively. But in the study, the controls used were healthy women, as well as the age-appropriate early-onset BC limit was <35 years. There is no similar study that discusses the relationship of early onset of breast cancer and FGFR2 polymorphism in other populations, but a study by Liang (Liang et al., 2008) found that there is a strong association of TT mutant genotypes and T alleles in FGFR2 polymorphism rs2981582 as risk factors of occurrence of BC in premenopausal women.

Our study indicates that the A allele of P21 and the allele T of FGFR2 may be linked to increased risk of early-onset BC in Yogyakarta, Indonesia. In order to confirm the findings, further analyses are needed.

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