Aim: to determine signaling pathways in breast cancers from patients aged 35 years old or younger and patients aged more than 35 years old.

Methods: this was a cross-sectional, comparative study of female breast cancer patients who were recruited and divided into two age groups, i.e. 35 years or younger and more than 35 years old. Specimens were obtained by biopsy or surgical removal of the tumors and were confirmed by histopathological examination. The expression of ER, IGF-1R, Her-2, MAPK, and cyclin D1 were measured using immunohistochemistry.

Results: ninety-three patients were recruited from September 2004 to December 2005. Forty-three patients were 35 years or younger. More than 90% of the patients within the two groups showed invasive ductal carcinomas and more than half of these tumors were grade 2. Immunohistochemical staining was successfully done in 90 patients. ER-alpha expression was negative in 33 breast cancers (78.6%) from patients less than 35 years old and 32 cancers (66.7%) of older patients. The expressions of IGF-1R, Her-2, MAPK, and cyclin D1 were positive, respectively in 17 (40.5%), 11 (26.2%), 28 (66.7%), and 7 (16.7%) cancers within the group of patients 35 years old or younger, and, respectively in 18 (37.5%), 11 (22.9%), 37 (77.1%), and 9 (18.8%) of cancers from patients more than 35 years old.

Conclusion: there were no statistically significant differences in the expression of any of the biomarkers between the two groups. In all patients, ER was negative in 72.2% cases and MAPK was positive in 76.7% cases. Patients aged 35 years or younger showed similar ER, IGF-1R, Her-2, MAPK, and cyclin D1 expressions compared to cancers from patients more than 35 years old.

key words: breast cancer, signaling pathway, ER, PR, Her-2, MAPK, cyclin D1.
carcinogenesis between patients aged 35 years or younger and patients aged more than 35 years.

METHODS

Study Design
This was a cross-sectional, comparative study of sporadic breast cancer patients aged 35 years or younger and patients more than 35 years old. The study was conducted at Dharmais National Cancer Center, the Division of Hematology-Medical Oncology, Department of Internal Medicine, University of Indonesia, between September 2004 and December 2005.

Recruitment of Study Subjects
The study populations were patients aged 35 years or younger and patients more than 35 years of age. Study subjects were recruited consecutively between September 2004 and December 2005. Inclusion criteria were patients with histopathologically confirmed carcinoma of the breast and gave their consents after sufficiently informed about the study. Exclusion criteria were a history of breast or ovarian cancers in the patient’s family, that is the patients’ siblings, mother, mother’s sisters or grandmother; patient suffering from liver disease or diabetes mellitus; patients having received chemotherapy (for pre-menopausal patients); pregnancy and lactation; and rejection from patients or her families to participate in the study. Once diagnosis had been histopathologically confirmed to be malignancy, patients were interviewed by investigator to obtain their family histories.

Immunohistochemistry
Specimens were paraffin-embedded tissues from biopsy or surgery of the breast tumors for immunohistochemical staining of ER, IGF-1R, Her-2, MAPK, and cyclin D1. Sections were cut to 4 mm thickness, mounted onto the silanized slides and allowed to dry overnight. Specimens were then deparaffinized with xylol and serial alcohol treatment in decreasing concentrations and then rehydrated. Blocking of endogen peroxidase was done by treating the sections with 0.5% H2O2 in methanol for 30 minutes. Slides were placed in a jar containing 10 mM sodium citrate solution (pH 6.0) and microwaved for antigen retrieval. Sections were isolated using a PAP pen (dakopatts) and incubated in phosphate buffered saline (pH 7.4) for 5 minutes. Then, the specimens were blocked with 3% normal horse serum for 20 minutes.

Immunostaining
Slides were incubated with primary antibody against the ER (rat monoclonal anti-ER, NCL-ER-6F112/2, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) diluted 1:60; the IGF-1R (RB-9240-R7, LabVision, Fremont, CA, USA), diluted 1:40; the Her-2 (NCL-c-erbB-2-316, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) diluted 1:80; the MAPK (NCL-MKK4, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), diluted 1:40; and the cyclin D1 (NCL-CylinD1, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) diluted 1:20. The slides were then immersed in normal horse serum for 10 minutes prior to antibody treatment and then incubated with primary antibody overnight in humidified chamber. After washing with TBS buffer, antibody binding was detected by incubation with biotinylated secondary antibody for 30 minutes in humidified chamber. Then the slides were rinsed with TBS, and incubated with streptavidin-horse radish peroxidase. Finally, the slides were washed with TBS and counterstained with hematoxylin.

Readings of Slides
Positive and negative controls were included for each staining. Positive staining showed brown color to the cells. For ER and cyclin D1 showed nuclear staining, whereas positive IGF-1R showed brown membrane and cytoplasmic staining. Her-2 positive staining was localized to the membrane, while MAPK positive staining was cytoplasmic. Validity of staining procedure was determined by including two slides of positive and negative controls. Positive controls for ER, IGF-1R, Her-2 and cyclin D1 were breast tissues, whereas testis tissues were used as positive control for MAPK expression. Slide where primary antibodies were omitted served as negative controls. ER expression was detected by light microscopy (magnification x 1000) and using the counting chamber in ocular lens with emersion oil on objective lens. Random counting was carried out on 1000 epithelial cells and the percentage of stained cells calculated. Intensity of staining was divided into 4 groups: 0: no staining; 1: weak staining, 2: moderate staining, 3: strong staining. Inter-observer variation was assessed using kappa statistics.

Data Management and Analysis
Patients’ characteristics and all variables were presented descriptively. Differences between patients aged 35 years or younger and patients more than 35 years were analyzed using the Chi-square or Fisher’s exact tests. A p value of less than 0.05 was considered significant. Analysis was done using the STATA version 8.0 software (STATA Corporation, TX, USA).
RESULTS

Patients’ Characteristics

Patients were consecutively included in this study. The total number of patients was 93, 43 patients were 35 years old or younger. The youngest patient was 24 years old and the oldest woman was 75 years old. Approximately 42% patients were post-menopausal women. The mean body mass index (BMI) was normal in both groups of patient. There was no significant difference in BMI between patients 35 years old or younger and those more than 35 years old. (Table 1)

Immunohistochemistry staining was successfully carried out in 90 specimens; three specimens were not usable. More than two-thirds of the cancers did not show detectable ER staining. There was no difference in immunostaining between patients 35 years old or younger and those more than 35 years old. (Table 5)

Tumor characteristics

Approximately 50% of the patients were diagnosed at early stages of the disease (I-II) with small-size tumors. Ipsilateral lymph node involvement may have been present. (Table 2) Invasive ductal carcinoma was the most common histopathological type diagnosed and approximately 50% of the patients showed grade 2 tumors. (Table 3 and Table 4) There was no significant difference between the two groups of patients in terms of tumor characteristics.
DISCUSSION

More than 50% of the patients were diagnosed at the early stages of the disease which is very different compared to more than 10 years ago, when patients were diagnosed at already advanced stages of the disease (III-IV). There was no difference between the two groups of patients in terms of tumor size (T), nodal involvement (N), metastasis (M), stages, and tumor grades. Similar results have also been reported by other study.24 Invasive ductal carcinoma was also the most frequent histopathological type of the tumors (88%-92.3%).30

The purpose of this study was to determine if differences in biological behavior that existed in sporadic breast cancer of young patients compared to older patients. We hypothesized that breast cancers of younger patients would show a more aggressive behavior compared to cancers of the older women and this would explain the high prevalence of young, non-familial, breast cancer patients at DNCC. Many studies from USA and Europe support this hypothesis.22-26

Unexpectedly, we found no differences between the two groups of study subjects in any of the parameters that were assessed. Although we found high number of ER-negative breast cancer cases in the group of young patients, a high proportion of ER-negative cancers also occurred in the older group. These results would suggest a high percentage of hormone independent breast cancers in this study population. The proportion of ER negative cases in both groups was substantially higher than reported by most other studies. Particularly notable is the fact that ER-negative breast cancers were two times more common in the study presented here compared to studies from USA and Europe. However, some studies from Asia have also found similar number of ER-negative breast cancer cases among their patients as described here. In Singapore, ER negative breast cancers were found in 59.2% of Malayan ethnic and 31.1% in non-Malayan ethnic women.25 In Jordan, ER-negative cases were found in 42% of patients less than 50 years old compared to 32% in patients aged 50 years.

| Table 5. Results of immunohistochemical staining |
|-----------------|-----------------|-----------------|-----------------|
|                | ≤ 35 years      | > 35 years      | P Value         |
|                | N (%)           | N (%)           | Chi square      |
| ER expression  |                 |                 |                 |
| Positive – weak| 4 (9.5)         | 4 (8.3)         | 8 (8.9)         |
| Positive – moderate| 0          | 2 (4.2)         | 2 (2.2)         |
| Positive – strong| 5 (11.9)     | 10 (20.8)       | 15 (16.7)       |
| Negative       | 33 (78.6)       | 32 (66.7)       | 65 (72.2)       |
| Total (%)      | 42 (100)        | 48 (100)        | 90 (100)        |
| IGF-1R expression|               |                 |                 |
| Positive – weak| 4 (9.5)         | 5 (10.4)        | 9 (10.0)        |
| Positive – moderate| 7 (16.7)     | 4 (8.3)         | 11 (12.2)       |
| Positive – strong| 6 (14.3)      | 9 (18.8)        | 15 (16.7)       |
| Negative       | 25 (59.5)       | 30 (62.5)       | 55 (61.1)       |
| Total (%)      | 42 (100)        | 48 (100)        | 90 (100)        |
| Her-2 expression|               |                 |                 |
| Positive – weak| 2 (4.8)         | 2 (4.2)         | 4 (4.4)         |
| Positive – moderate| 2 (4.8)      | 1 (2.1)         | 3 (3.3)         |
| Positive – strong| 7 (16.6)      | 8 (16.6)        | 15 (16.7)       |
| Negative       | 31 (73.8)       | 37 (77.1)       | 68 (75.6)       |
| Total (%)      | 42 (100)        | 48 (100)        | 90 (100)        |
| MAPK expression|                 |                 |                 |
| Positive – weak| 4 (9.5)         | 4 (8.3)         | 8 (8.9)         |
| Positive – moderate| 14 (33.3)    | 23 (47.9)       | 37 (41.1)       |
| Positive – strong| 10 (23.9)     | 10 (20.9)       | 20 (22.2)       |
| Negative       | 14 (33.3)       | 11 (22.9)       | 25 (27.8)       |
| Total (%)      | 42 (100)        | 48 (100)        | 90 (100)        |
| Cyclin D1 expression|           |                 |                 |
| Positive – weak| 2 (4.8)         | 1 (2.1)         | 3 (3.3)         |
| Positive – moderate| 1 (2.4)      | 2 (4.2)         | 3 (3.3)         |
| Positive – strong| 4 (9.5)        | 6 (12.6)        | 10 (11.1)       |
| Negative       | 35 (83.3)       | 39 (81.2)       | 74 (82.3)       |
| Total (%)      | 42 (100)        | 48 (100)        | 90 (100)        |
or older. In Korea, 58.9% ER-negative breast cancers were found in women 35 years old or younger compared to 48.2% ER-negative cases in women older than 35 years. These studies seem to indicate that ER-negative breast cancers are more common in women from Asia than in women from USA and Europe.

Some conditions might have caused the negative staining of the ER protein. First, there could be mutations in the ERα gene resulting in clinically classifying the cancers ER-negative and hormone-resistant tumors. Second, the loss of ER expression could have resulted from aberrant methylation on the 5'CpG island of ER gene prior to the acquisition of invasiveness of the cancers. Third, loss of transcription factor(s) that are essential for the transcription activity of the ERα gene has also been reported. These mechanisms that cause loss of ER expression have been suggested to occur in a small number of ER-negative breast cancer cases in USA and Europe and are associated with tumor aggressive phenotype.

Recent studies have shown that ERα is associated with the cell membrane and activates intracellular signaling termed membrane-initiated steroid signaling (MISS). This relative new insight into how the ER might act may be clinically important. ER does not act alone in stimulating tumor growth, but there is a complex interacting network between the ER and growth factor signaling to ensure the survival of cancer cells. Translocation of the membrane-associated ER appears to interact with both Shc and IGF-1R. It is likely that IGF-1 is an important factor involved in tumor growth promotion in the presence of ERα and might be activated via Raf-Ras-MAPK signaling pathway. In vitro study of ER-expressing breast cancer cell line (MCF-7) has shown that estrogen and IGF-1 are mitogens that can communicate with each other through their receptors and act in normal or cancerous cells.

The administration of antiestrogen decreases not only the estrogen/ER activation but also the IGF-1 pathway. Membrane-associated ERα may interact directly with IGF-1R and cause IGF-1R activation through MAPK. However, crosstalk between IGF-1R and ER was shown in ER-expressing cell line. Other studies have also shown that growth factors are capable of crosstalk with membrane-associated ER, an activity that results in higher proliferation rate. Bidirectional crosstalk might occur between ER and HER-2 or EGFR that could potentiate both receptors leading to resistance of anti-hormonal (Tamoxifen) therapy. Communication between ER and growth factors also takes place in the cell membrane. The MAPK/extracellular signal regulated kinase (ERK) system has been shown to be the downstream signaling cascade leading to the activation of major cell cycle proteins, such as cyclin D1 and cyclin E and the transcription factor c-myc.

The existence of ER-membrane signaling has changed our paradigm on the role of ER in breast cancer progression and hormonal resistance. Identification of membrane ER should be a routine practice in future assessment of the ER status in breast cancer, since current immunohistochemistry (IHC) technique detects only ER that resides in the nuclei of cancer cells. It would also be interesting to determine, whether breast cancer cells that have been determined ER-negative by IHC staining contain significant membrane ER. If this were the case, it could explain the aggressive nature of these breast tumors which would be utilizing the membrane ER and crosstalk with growth factor receptors.

In this current study, IGF-1R expression was not detected in 59.5% and 62.5% of breast cancers from patients <35 years old and >35 years, respectively. These results indicate that IGF-1 signaling pathways are not dominant in the cancers of the study subjects and support the hypothesis of close interaction between ER and IGF-1R as high percentage of the cancers are also ER negative. Our study also showed high MAPK activation in the two groups of patients. This indicates that the majority of the cancers in these patients rely on the growth factor signaling pathway rather than hormonal (ER) signaling pathway for their growth. The result may indicate that a high percentage of the cancers in these patients were membrane-associated ER, but further studies are needed to confirm this hypothesis. Her-2 expression was shown in more than 20% of the cancers in both groups. This is similar to what has been reported in other study, that is, 20-30% Her-2 positive could be expected in breast cancer, especially in young patients. Overexpression of Her-2 is associated with more aggressive cancer and resistance to chemotherapy. It has been proposed that breast cancers should be classified into 4 major groups, i.e. Her-2 positive, Her-2 negative, ER-positive, and basal-like tumors that do not express either Her-2 or hormone receptor. This classification may be useful in deciding which treatment plan would be most effective for each individual case.

**CONCLUSION**

This study shows no difference in the expression of ER, IGF-1R, Her-2, MAPK, and cyclin D1 in cancers of young breast cancer patients (35 years old or younger) and older patients. The hormonal (ER) signaling...
pathway was not dominant in cancers of these women, as close to two-thirds of the cancers were ER-negative. A high percentage of these cancers were also negative for IGF-1R expression, perhaps indicating interaction between ER and IGF-1R. A large number of these cancers showed a high expression of MAPK, indicating that growth factor signaling pathways were dominant in the carcinogenesis of these breast cancers.

REFERENCES


