The Expression of NFATc1 is more Predominant in Triple Negative Breast Carcinoma Patients

Riana Sari Puspita Rasyid1, Krisna Murti2, Zen Hafy1

1Departemen Histologi, Fakultas Kedokteran, Universitas Sriwijaya, Palembang
2Departemen Patologi Anatomi, Fakultas Kedokteran, Universitas Sriwijaya, Palembang

rianasaripuspita@fk.unsri.ac.id
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Abstract

Breast carcinoma is the most common malignancy among women in developed and developing countries. Invasive breast carcinomas are classified into 4 subtypes i.e. luminal A and B, human epidermal growth factor receptor 2 (HER2)-enriched and triple negative, with the latter having the worst prognosis. Nuclear factor of activated T cell (NFATc1) is an important transcription factor in malignant transformation and progression. Therefore, NFATc1 expression may determine prognosis of breast carcinoma. This study aimed to determine the roles of NFATc1 in breast carcinoma progression. Materials and methods, fifty-two paraffin blocks were selected and prepared to assess NFATc1 expression by immunohistochemistry. These data were taken from medical records: i.e. molecular classification, patient age, tumor size, lymphovascular invasion and grade of tumor were recorded. Positive NFATc1 expression was observed in 4 samples i.e. in the nuclei of luminal A (1 out of 12; 8.8%), luminal B (1 out of 15; 6.7%), and triple negative (2 out of 12; 16.7%), but no NFATc1 expression was detected in HER2-enriched samples. Clinically, more of these patients were in the fifth decade (38.5%), with larger tumor size (≥2 cm; 90%), lymphovascular invasion positive (80.8%), and high degree (3; 59.6%). NFATc1 expression is more predominant in triple negative breast carcinoma.

Keywords: breast carcinoma, clinicopathology, immunohistochemistry, molecular subtype, NFATc1
1. Introduction

Breast carcinoma is the most common malignancy among women, both in developed and developing countries. Based on data from the World Health Organization (WHO) in 2012, in Indonesia breast carcinoma ranks first out of all malignancies in women, with 48,998 cases.1 Breast carcinoma is the fifth leading cause of death among women worldwide (6.4%) and the second leading cause of death in developed countries reaching up to 198,000 cases or 15.4%.2 In 2012, breast cancer was the first cause of death in Indonesia (40.3%) related to malignancy in women.1-3

Invasive breast carcinoma can be classified by molecular markers i.e estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/Erb2/Neu) and Ki67.4 Based on these markers, breast carcinoma is classified into 4 subtypes namely luminal A (ER+/PR+/high, HER2, Ki67<14%), luminal B (ER+/PR+/low, HER2+/HER2-, Ki67≥14%), HER2-enriched/over-expression (ER-/PR-,HER2+), and triple negative subtype (ER-, PR-, HER2-) also called the basal like tumors.4,5 This classification can be used to evaluate the prognosis of breast carcinoma. The prognosis of breast carcinoma is determined by many factors. Prognosis and predictive factors affecting breast carcinoma include patient’s age at time of diagnosis, tumor size, lymph node status, histopathologic type and grade, as well as ER, PR or HER2 status.6

Nuclear factor of activated T-cells (NFAT) is an important transcription factor in physiology and pathology in malignant transformation and progression.7 Active NFAT pathways can lead to tumor development (tumorigenesis), invasion and metastatic processes of breast tumor cells.3 In the cytoplasm, an inactive state of NFAT is in a strongly phosphorylated condition.7 NFAT activation begins with increased Ca2+ in the cytoplasm. Subsequently, calcium binds to calmodulin (CAM) and activates calcineurin (CN) phosphatase enzyme. NFAT undergoes dephosphorylation by CN. After most of the phosphate has been removed, NFAT translocates to the nucleus and induces gene transcription of many genes, including cytokines and genes of cellcycles regulation. NFAT activation leads to proliferation, angiogenesis, metastatic processes, and progressivity of carcinoma. Previous study showed that nuclear localization of NFATc1 was reported to be associated with worse prognosis and the prevalence of NFATc1 was found to be the highest in triple negative subtype breast carcinoma about 50.6%.3 Other study also demonstrated that NFAT was associated with development of breast carcinoma cells.8

The high incidence and high mortality rate of breast carcinoma indicate the importance of determining prognostic factors of every new case of breast carcinoma to save the life of patients. This study will determine the role of NFATc1 protein expression in progressivity of invasive breast carcinoma patients in the Department of Anatomic Pathology Faculty of Medicine Universitas Sriwijaya/RSUP Dr. Moh. Hoesin Palembang. From this study we hope to establish the development of diagnostic and prognostic biomarkers as well as therapeutic candidates for breast carcinoma.

2. Methods

This study was approved by the Health Research Ethics Committee of RSUP Dr. Mohammad Hoesin Palembang and Medical Faculty of Universitas Sriwijaya No. 394/kepkrsmhfikunsri/2017.

An observational analytic study with cross sectional design has been carried out from August 2017 until February 2018 in Department of Anatomical Pathology Faculty of Medicine, Universitas Sriwijaya/RSUP Dr. Moh. Hoesin Palembang, Indonesia. The size of the sample was determined by hypothesis test the proportions of two populations with the minimum sample size required was 52 paraffin blocks. The sampling technique used in this study was disproportionate stratified random
The histology and immunostaining were evaluated by a pathologist. Positive NFATc1 expression was considered when we saw brown color in at least a single nucleus of tumor cells, regardless the intensity of brown color either weak, moderate, or strong.

2.3. Data analysis
The relation between dependent variable and independent variable was determined by Chi Square statistical test ($X^2$) with confidential interval 95% and value of $α=0.05$. Data were analyzed by SPSS ver. 21 (IBM Corporation, New York, USA).

3. Results
3.1. Clinicopathological data
Among 52 subjects, there were 28.8% of luminal B, 25% of HER2-enriched, while triple negative and luminal A have similar value i.e. 23.1% (Table 1). More subjects were in fifth decade (38.5%) and with larger tumor size ($≥2$ cm:90%). Most of the subjects (80.8%) showed positive lympho-vascular invasion (LVI). In addition, more subjects were observed having high grade (grade 3:59.6%).

Prognosis of breast cancer patient can be indirectly determined by molecular subtype i.e. good prognosis (luminal A and luminal B) and poor prognosis (HER2-enriched and triple negative). Based on this classification, our cohort shows that slightly more patients have a good prognosis i.e. 51.9 % (Table 2).

There was no significant relation between NFATc1 protein expression and molecular subtypes, age of the patient, tumor size, LVI, and the tumor grade (Table 1). There also was no significant correlation between patient prognosis and NFATc1 protein expression (Table 2). However, we observed a significant correlation between molecular subtypes with tumor grade (Table 2; $p$ value=0.002).

2.2. NFATc1 immunostaining evaluation

The paraffin blocks were cut with a thickness of 4 μm (one layer of tissue) using a microtome and attached to a poly-L-lysine coated glass. The paraffin blocks were deparaffinized with xylene before they were finally dehydrated. Endogenous peroxide activity was blocked by incubating the slides with hydrogen peroxide. Microwaving the slides in 10 mM citrate buffer (pH 6.5) will completely retrieve the antigen before incubating it in a blocking solution. Furthermore, the slides were incubated with mouse monoclonal NFATc1 antibody, clone 7A6 for 1 hour at room temperature (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After washing, the slides were then incubated with trakkie Universal Link (ScyTek Laboratories, West Logan, UT, USA) in blocking solution, subsequently by 1:500 diluted horseradish peroxidase labeled streptavidin (ScyTek Laboratories). Sections were stained with 50 mg/ml diaminobenzidine tetrahydrochloride (DAB) and 0.06% hydrogen peroxide, then counterstained in 1:10 diluted Lillie-Mayer Haematoxylin, dehydrated in sequential absolute ethanol, repeated clearing with xylene and subsequently covered by glass cover slips. A tissue section of Chondroblastoma case was used as a control.

Tissue sections were captured by olympus BX51 (Olympus, Tokyo, Japan), dual-headlight microscope equipped, an Olympus Q-Color 5 digital camera with a 40 plan-apochromat objective. Digital images were obtained and adjusted using Olympus DP21 (Olympus).

2.1. NFATc1 Immunohistochemistry
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Table 1. Association between NFATc1 expression with molecular subtype and clinicohistopathology characteristics

| Variable                  | NFATc1 Expression | Total | *p value |
|---------------------------|-------------------|-------|----------|
|                           | Negative n (%)    | Positive n (%) |       |
| Subtypes                  |                   |       |          |
| Luminal A                 | 11 (91.7)         | 1 (8.3) | 12 | 0.368 |
| Luminal B                 | 14 (93.3)         | 1 (6.7) | 15 |
| HER2-enriched             | 13 (100)          | 0 (0)  | 13 |
| Triple negative           | 10 (83.3)         | 2 (16.7)| 12 |
| Age (years)               |                   |       |          |
| < 30                      | 3 (100)           | 0 (0)  | 3  |
| 30 - 39                   | 10 (100)          | 0 (0)  | 10 |
| 40-49                     | 13 (92.9)         | 1 (7.1) | 14 |
| 50-59                     | 17 (85.0)         | 3 (15.0)| 20 |
| 60-69                     | 4 (100)           | 0 (0)  | 4   |
| 70-79                     | 1 (100)           | 0 (0)  | 1   |
| **Tumor Size**            |                   |       |          |
| ≥ 2cm                     | 24 (88.9)         | 3 (11.1)| 27 | 0.360 |
| < 2cm                     | 2 (66.7)          | 1 (33.3)| 3   |
| LVI                       |                   |       |          |
| Positive                  | 38 (90.5)         | 4 (9.5)| 42 |
| Negative                  | 10 (100)          | 0 (0)  | 10 |
| Tumor Grade               |                   |       |          |
| Grade 3                   | 28 (90.3)         | 3 (9.7)| 31 |
| Grade 2                   | 20 (95.2)         | 1 (4.8)| 21 |
| Prognosis                 |                   |       |          |
| Poor                      | 23 (92.0)         | 2 (8.0)| 25 |
| Good                      | 25 (92.6)         | 2 (7.4)| 27 |

*Chi Square test
**The information of tumor size was observed only on the medical records of 30 samples among 52 total samples

Table 2. Association between molecular subtype with clinicohistopathology characteristics

| Variable                  | Molecular Subtypes |
|---------------------------|--------------------|
|                           | Luminal A n (%)    | Luminal B n (%) | HER2-enriched n (%) | Triple negative n (%) | Total n (%) | *p value |
| Age                       |                   |               |                   |                     |             | 0.525    |
| <30                       | 1 (33.3)          | 1 (33.3)      | 1 (33.3)          | 0 (0)               | 3            |
| 30-39                     | 2 (20.0)          | 4 (40.0)      | 3 (30.0)          | 1 (10.0)            | 10           |
| 40-49                     | 3 (21.4)          | 2 (14.3)      | 6 (42.9)          | 3 (21.4)            | 14           |
| 50-59                     | 4 (20.0)          | 8 (40.0)      | 2 (10.0)          | 6 (30.0)            | 20           |
| 60-69                     | 2 (50.0)          | 0 (0)         | 1 (25.0)          | 1 (25.0)            | 4            |
| 70-79                     | 0 (0)             | 0 (0)         | 0 (0)             | 1 (100)             | 1            |
| **Tumor Size**            |                   |               |                   |                     |              | 0.359    |
| ≥ 2 cm                    | 6 (22.2)          | 8 (29.6)      | 5 (18.5)          | 8 (29.6)            | 27           |
| < 2 cm                    | 0 (0)             | 2 (66.7)      | 0 (0)             | 1 (33.3)            | 3            |
| LVI                       |                   |               |                   |                     |              | 0.578    |
| Positive                  | 9 (21.4)          | 11 (26.2)     | 11 (26.2)         | 11 (26.2)           | 42           |
| Negative                  | 3 (30.0)          | 4 (40.0)      | 2 (20.0)          | 1 (10.0)            | 10           |
| Tumor Grade               |                   |               |                   |                     |              | 0.002    |
| Grade 3                   | 5 (16.1)          | 8 (25.8)      | 6 (19.4)          | 12 (38.7)           | 31           |
| Grade 2                   | 7 (33.3)          | 7 (33.3)      | 7 (33.3)          | 0 (0)               | 21           |

*Chi Square test
**The information of tumor size was observed only on the medical records of 30 samples among 52 total samples
3.2. Immunohistochemical Analysis

As previously reported, NFATc1 is an important transcription factor for osteoclast activation, hence NFATc1 was highly expressed by osteoclast. Therefore, in this study we used a chondroblastoma case as a control (Figure 1A). From 52 samples, NFATc1 protein expression was positive in 4 subjects (7.7%).

In this study, different NFATc1 staining patterns were observed. In the luminal A, NFATc1 expression was only seen in tumor cells that were disassembled in small clusters at the peripheral tumor of larger clusters (Figure 1B). While in luminal B (Figure 1C), NFATc1 expression was mostly observed from weak to moderate expression in the center of a large tumor cluster. In the triple negative groups, expression of NFATc1 protein was observed in the tumor nuclei, from weak, moderate to strong expression, especially in larger and denser tumor clusters, both in tumor cells at the center and periphery of large tumor clusters (Figure 1D and 1E). The positivity of the nuclei corresponded to the positive control showing NFATc1 expressed in osteoclasts (Figure 1A). In the HER2-enriched group, no sample expressing NFATc1 protein was found (Figure 2).

FIG 1. Immuno-expression of NFATc1 protein. (A) Expression of NFATc1 protein in nuclei of osteoclast (arrow, positive control). (B) In luminal A, the nuclei of tumor cells with positive NFATc1 expression were seen only in disassembled tumor cells or in small groups at the peripheral of larger tumor clusters. (C) In luminal B, NFATc1 protein expression appears in the nuclei of tumor cells, especially in the denser tumor group. (D & E) Positive NFATc1 expression in nuclei of denser tumor clusters of the triple negative group.
4. Discussion

In this study, we observed that there were more patients in the fifth decade (38.5%) with the median age of 47 years. Similar to other studies, the median age of breast carcinoma patients at the time of diagnosis was 52 years in University Malaya Medical Center (UMMC) Malaysia and 47 years in Dharmais Cancer Center (DCC) Indonesia. It supports the fact that increased age is a risk factor of breast cancer.

The majority of breast cancer cases among Indonesian women is usually found in a more advanced stage. Similar to other data in developing countries, our data showed that most cases of breast carcinoma were observed in patients with larger tumor size, at advanced tumor grade and with positive LVI.

Despite that there was no significant association between NFATc1 protein expression with tumor size, tumor grade, and LVI, positive NFATc1 expression were more commonly (3 out of 4; 75%) found in larger tumorsize (≥2 cm), in more advanced tumor grade (3 out of 4; 75%), and with positive LVI (100%). This fact is similar to the previous studies which suggest that NFATc1 affects the invasive and metastatic processes as well as the proliferation and progression of breast carcinoma. Activation of NFAT via calcium signaling is initiated by cell surface receptors such as T-cell receptors (TCR), receptor tyrosine kinases (RTKs) or G-protein receptors. In this context, the mechanism of NFAT activation has been largely deduced based on studies of cellular immune cells. However, the same mechanism of activation and function of NFAT also occurs in the cells comprising the tumor and the microenvironment, especially endothelial cells. The underlying event in NFAT activation is an increase in intracellular calcium which also affects other cancer-associated pathways initiated by cell surface receptors that stimulate the activation of phospholipase type C (PLC) enzymes such as PLCγ. Efficient NFAT activation also requires sustained calcium signaling, and this is achieved by the opening of calcium-release activated channels (CRAC) in the plasma membrane. This occurs in response to calcium emptying initiated by PLCγ from the endoplasmic reticulum (ER). Then, calcium release occurs due to the high affinity of ER calcium for the stromal interaction molecule (STIM1) which causes a conformational change in the Orai 1 protein CRAC channel, opening the channel and leading to extracellular calcium influx. After undergoing continuous flux through the ER and calcium channels, calcium then binds to calmodulin, leading to binding and activation of serine/calcineurin phosphatase. NFAT will be dephosphorylated (the process of withdrawing a phosphate group) by CN. After most of the phosphate has been released, NFAT is then translocated to the cell nucleus to induce the transcription of genes including cytokines and cell cycle regulatory genes. Given the important role of calcium in the activation of NFAT in the cytoplasm of cells in cellular immunity cells, the role of NFAT in carcinoma development is also affected by calcium influx. Consistent with this, calcium
signaling influences tumor cell proliferation and invasive migration. In the migration of immune cells into the tumor microenvironment, calcium controls cell polarity and cytoskeletal remodeling. This NFAT reaction causes proliferation, angiogenesis, metastasis and inflammation of a carcinoma.  

We also observed a statistically significant association between molecular subtypes and tumor grades, where all patients of the triple negative group had an advanced tumor degree (100%). This group has the worst prognosis among other subtypes and is usually characterized by histologic features of advanced tumor grade. This finding is in line with a previous study that suggested the triple negative subtype is characterized by advanced tumor grade features.  

Related to NFATc1 expression, we noticed that the two samples of luminal subtypes with positive NFATc1 protein expression have their own characteristics. 

Luminal A group with positive NFATc1 protein expression were more likely to be found in a relatively younger patient (45 years), with large tumor size, positive LVI and grade 2. In this group, tumor cells that NFATc1 were only observed as separated single tumor cells and in small clusters, which located peripheral of a larger group. The expression of NFATc1 was not found in the large clusters. This observation demonstrates that tumor cells with NFATc1 expression most likely represents ongoing invasion in nearby or distant areas. It was also possible that the group of luminal subtype A showing NFATc1 protein expression is a variant of luminal A subtype that tends to be more aggressive and has a worse prognosis than other luminal A subtypes. This suggested that luminal A may be separated into good and worse prognosis subtypes based on NFATc1 expression. This possibility should be confirmed by further experiment. 

In the luminal B subtype the subjects with positive NFATc1 protein expression, the pattern was similar to that in triple negative group. Patients in this group with NFATc1 positive expression were detected among older age, with large tumor size, positive LVI, and advanced tumor grade with high Ki-67 expression. Ki-67 expression is closely related to cell cycle and mitosis. The high expression of Ki-67 in this sample signified that tumor cells tend to be more proliferative and were associated with progression and a poorer prognosis of breast carcinoma. This finding may suggest that tumor cells with NFATc1 expression among Luminal B subtype have a worse prognosis compared to the others without NFATc1 expression. To verify this premise more investigations should be conducted. 

In the triple-negative group with positive NFATc1 expression, the patient’s age in both samples were older than other samples of triple negative’s group (52 and 53 years), tumor size varies (<2 cm and ≥2 cm), both were accompanied by positive LVI and advanced tumor grade. NFATc1 protein was moderate to strongly detected in the nuclei expressed in large groups of tumor cells in the center to the periphery of the clusters, unlike those seen in the luminal A (figure 1D and 1E). Our finding is in accordance with other study where positive NFAT protein expression is most prevalent in triple negative subtype breast carcinoma. Although the percentage of our findings is lower than in the data of other study, which is possible due to the limited number of our samples particularly in the triple negative subtype. 

In the HER2-enriched group, there was no sample found which expressed NFATc1 protein (figure 2). One of the possible mechanisms responsible for this event is the presence of epigenetic silencing in genes that affect the expression of NFATc1 proteins, such as histone modifications and DNA methylation events in the NFATC1 promoter. To prove this possibility further research such as DNA methylation assays are required. In addition, patients with HER2 expression usually have worse prognosis, but, with appropriate therapeutic management including
administration therapy with anti-HER2 combined with chemotherapy will improve patient’s survival rate.\textsuperscript{17} It is possible that the negative NFATc1 expression in patients from the HER2-enriched subtype group has a predictive or prognostic meaning. Further research is necessary to prove this finding.

The limitation of this study is the particularly smaller sample size when classified into 4 subtypes. The sample should be extended for the next study and enable us to focus on each subtype.

5. Conclusion

NFATc1 expression is more predominant in the worse prognosis of subtypes, triple negative breast carcinoma, hence, it is most likely NFATc1 expression is important for progression of breast carcinoma.

Several other types of malignancy can be found with positive expression of NFATc1 protein in the nucleus, this is caused by continuous translocation of NFATc1 (constitutive activation) which then interacts with other transcription factors; the most frequently found is the MYC transcription factor.\textsuperscript{18,19} MYC is a proto-oncogene that plays an important role in cell cycle regulation.\textsuperscript{20} Mutations cause the MYC proto-oncogene to become an oncogene.\textsuperscript{21} The interaction between the continuously active NFATc1 and MYC induces MYC deregulation, which includes MYC amplification.\textsuperscript{22} MYC amplification can result in decreased expression of tumor suppressor genes and increased oncogene activity.\textsuperscript{23} The increased activity causes continuous cell cycle activation, so that breast tumor cells proliferate continuously, the progression of breast tumor cells increases which results in a poor prognosis in these breast cancer patients.\textsuperscript{20} Based on this it can be concluded that positive NFATc1 expression is usually associated with continuous NFATc1 activation and is usually found in breast carcinomas with poor prognosis.

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Conflicts of Interest

The authors affirm no conflict of interest in this study.

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