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INTRODUCTION

Breast cancer (BC) is the most common form of cancer in women and the primary cause of cancer-related deaths among women. The two most prevalent types of BC are ductal type (IDC), which accounts for 80% of all cases, followed by lobular type (ILC), which represents 10% of BC patients. If cancer cells can move and endure in the face of the body's defenses and chemotherapy medications, BC's metastatic phase may occur. In order to survive and spread, cancer cells must also be able to escape the apoptotic process by making proteins to defend themselves and altering signaling pathways to prevent it from happening. In ILC, round cells with scant pale cytoplasm surround the breast tissue by infiltrating the stroma in a single pattern. Infiltration usually does not cause severe damage to anatomic structures around cells or trigger a massive connective tissue response. Due to its characteristic histological pattern, lobular carcinoma is sometimes challenging to diagnose by clinical examination and can make early diagnosis more difficult; ILC patients usually come at advanced stages.

Two options for surgical procedures for early-stage BC are Breast Conserving Surgery (BCS) and Modified Radical Mastectomy (MRM). BCS was a wide tumor excision with ipsilateral axillary or sentinel node dissection. Modified radical mastectomy was a procedure to remove the tumor along the skin over the mammary gland, accompanied by axillary dissection performed simultaneously. Even though the entire breast was removed during the MRM operation, recurrence and metastases are still frequent. The peak time for recurrence was the first two years after the primary surgery procedure. Chemotherapy might be given to decrease the recurrence possibility. In order to improve treatment efficacy and lessen adverse effects, the chemotherapy regimen typically employs a multi-drug regimen. The most widely used chemotherapy regimens for breast cancer are combinations of anthracycline-based and taxane-based regimens.

Cancer cells underwent the epithelial-to-mesenchymal transition (EMT) to

ABSTRACT

Background: Lobular type breast cancer (ILC) was the second most common type after ductal breast cancer. Chemotherapy may be used to lower the risk of recurrence in addition to surgery, which is the main treatment for early-stage breast cancer. Apoptosis is one of the body's techniques for killing cancer cells; thus, cancer cells must be able to evade it to survive. Due to the rarity of the disease, it is uncommon for researchers to investigate ILC, especially at an early stage. This study aimed to determine the mechanism of apoptosis evasion in ILC after mastectomy and chemotherapy who got local recurrence within two years after surgery by analyzing the expression of vimentin, IL 10, and CD 95.

Methods: This research was a cross-sectional study of medical records in a single center. The sampling method was total sampling, i.e., all patients underwent a mastectomy and received chemotherapy from January 2014 to December 2019 (5 years). Immunohistochemistry analysis was used to check the vimentin, α-IL10, and CD 95 levels in paraffin blocks from surgical tissues. A chi-square test and a path analysis with a linear regression test were the statistical tests used in this study.

Results: Seven samples contained local recurrence, while only six did not. According to statistical tests, the expression of vimentin, IL10, and CD95 between the recurrence and non-recurrence groups was significantly different. The chi-square test showed CD95 is a protective factor for local recurrence (odds ratio [OR] = 0.03, 95% confidence interval [CI] = 0.002-0.68 p =0.001). The multivariate analysis showed that vimentin expression has a significantly strong correlation with IL10 expression (p=0.000); the IL10 expression has a significant negative correlation to CD95 expression (p=0.03); and the CD95 expression was strongly correlated with the local recurrence (p=0.001).

Conclusion: Apoptosis evasion had an important role in the local recurrence of ILC after mastectomy and chemotherapy.
acquire immortality and the ability to invade and migrate. Cancer cells could transform from an immobile epithelial phenotype to a more mobile mesenchymal phenotype through this process.\(^8\) Cancer cells become more aggressive and resistant to chemotherapy during the EMT phase. The expression of several immunosuppressive cytokines, such as IL10 and TGF-β, increased due to the mesenchymal character. Mesenchymal tissue is looser and more diffuse than epithelial cells, does not have a clear polarity and is arranged in looser bonds, making them more flexible, individual, and motile. The most used tumor marker for the EMT process was vimentin. A study conducted by Panigoro et al. found that increased vimentin expression and decreased E-cadherin expression caused breast cancer patients to be resistant to chemotherapy drugs.\(^9\)

The immune system was believed to be significant in the recurrence process. Antitumor response and interaction between the patient’s immune system and cancer cells have received extra attention in managing BC and have become the subject of intense research over the last decades. It is known that several cytokines, including Interleukin-10 (IL10), play a crucial role in the coordinated recurrence of breast cancer.\(^10\) IL10 is a robust immune suppressant cytokine that inhibits the proliferation of T cells and their functions. Therefore, the cancer cells will release IL10 to assist them in their ability to inhibit immunological responses and thwart their eradication by the immune system of the host. Interleukin-10 made cancer cells resistant to the immune system and chemotherapy drugs by upregulating the Bcl-x ligand, hence preventing signaling that triggers apoptosis through the death receptor pathway. Long-held theories hold that the death receptor/death ligand system composed of CD95 (Fas/APO-1) and its ligand mediates the triggering of apoptosis to maintain immunological homeostasis.\(^11\)

Studies on ILC instances are infrequent since the ILC type is rarer than the ductal type, particularly those present at an early stage. This study intends to investigate the mechanism of local recurrence in early-stage breast cancer of the ILC subtype that has undergone adjuvant chemotherapy in terms of apoptosis evasion through the analysis of vimentin, CD95, and IL10 expression. The benefit of this research is that it can be used by a surgeon to manage breast cancer patients and educate patients and their families.

METHODS

Study design and subjects

This research was cross-sectional and involved analytical observation. Data were gathered from the Dr. H. Koesnadi Bondowoso Hospital’s medical records division in East Java, Indonesia. Between January 2020 and December 2022, data were gathered, examined, and analyzed. The Faculty of Medicine, Universitas Jember’s Research Ethics Committee has approved this study. Diagnoses of breast cancer were made using pathology and anatomy reports. Samples of the study were ILC patients who underwent mastectomy performed by the first author himself during January 2014 - December 2019, had adjuvant chemotherapy using a taxane and anthracycline base regimen and had local recurrence within two years after mastectomy. Total sampling was used to collect data from ILC patients who met the inclusion and exclusion criteria. The inclusion criteria of this study were early-stage ILC (stages I and II) who have carried out mastectomy and received adjuvant chemotherapy with taxane and anthracycline base regimens six times every three weeks (one cycle). The exclusion criteria of this study were: patients received external radiation therapy, another malignancy in other organs, pathology examination report stating that the edge of the resection was not tumor-free (less than two centimeters), paraffin blocks were damaged and could not be used. The cancer stage was obtained from clinical examination, radiology, and pathology reports. Pathological grading was evaluated by two pathologists based on the Bloom-Richardson grading system.\(^12\) After receiving an explanation, all patients who participated in this study signed a consent form to participate and a publication consent form.

Immunohistochemistry (IHC) staining and assessment

Paraffin derived from surgical specimens was examined using the immunohistochemical method. The vimentin, IL10 and CD95 monoclonal antibody used in this study came from the Mouse anti-Human CD95 Monoclonal Antibody from MyBioSource with the catalog number MBS475544 (vimentin), MBS704576(ILI0) and MBS10754069 (CD95). By counting the number of cells that responded favorably to the antibody on ten different fields of view under a 400x light microscope, the average levels of expression of vimentin, IL10, and CD95 were determined.

Statistical Analysis

Data was analyzed using OpenEpi 3.0.1 and EZR.\(^13\) Quantitative data were presented in mean ± SD (standard deviation). The normality test was done with the Shapiro-Wilk test and then analyzed with independent t-tests or Mann-Whitney. Pathway analysis used a logistic regression test. The statistical value that was considered significant was <0.05.

RESULTS

Subjects in this study were seven patients with local recurrence and six patients who did not have local recurrence. Vimentin, IL10 and CD95 expressions assessed were the averaged results of the number of cells that reacted positively to the antibody in ten fields of view of the light microscope using 400x magnification. Bivariate analysis revealed no significant differences in the variables of the two groups for clinical and pathological results based on age (mean), hormonal status (premenopausal and menopausal), grading, and lymph node metastasis (spread of cancer cells to the ipsilateral axillary lymph nodes). Detailed data from these results are presented in Table 1.

The statistical test used to compare variances in the expression of IL10 and CD95 was the Mann-Whitney non-parametric test, with test results were significant differences in IL10 and CD95 expression for the group with local recurrence and group without local recurrence (p=0.004 and p=0.045,
An Independent t-test was used for vimentin expression and showed a significant difference in mean expression for both groups (p=0.021). Table 2 displays the results of a statistical test based on the mean, standard deviation, and maximum-minimum values.

A logistic regression test was applied to the pathways correlation between each variable. The test result showed that vimentin expression has a significant correlation with IL10 expression (p=0.000) and has a strong correlation (β=0.670). The significant negative correlation obtained from the test between IL10 expression and CD95 expression (p=0.03, β=−0.600 respectively) indicates that IL10 expression decreases CD95 expressions. Strong correlations were shown between CD95 and local recurrence (p=0.001, β=−0.802); these results could be interpreted that the lower the expression of CD95, the greater the possibility of local recurrence. The detailed statistical test results are presented in Table 2, and the result from the pathway analysis is shown in Table 3 and Figure 1.

**DISCUSSION**

Recurrence after mastectomy has been well-known as the worst predictor for breast cancer patients. Several studies have shown that age, tumor size, histopathological grading, and hormonal status are significant local recurrence factors. Breast cancer in young women was more aggressive than in older women, and it had many other characteristics associated with a poor prognosis, such as high proliferation rates and illnesses of grades 3 and 4. Young individuals with breast cancer also have a higher chance of recurrence and a shorter disease-free survival. This study found no significant difference in the group with local recurrence compared to those without local recurrence. The small sample size was probably to blame for this outcome. Due to the high degree of heterogeneity in breast cancer, variations in the study’s sample size would impact the findings.

According to other histologic tumor features, young patients typically have high-grade and highly proliferative breast cancers, including lymph vascular invasion and Ki67 as disease biology indicators. Breast cancer recurrence was impacted by tumor size. Breast cancer is staged according to the size of the tumor, the involvement of the lymph nodes, and the presence of distant metastases. The most significant prognostic markers were lymph node metastases and tumor size. A rising lymph node metastasis was associated with an expanding tumor size. There was no discernible difference between the groups regarding lymph node metastasis and tumor size in this investigation. This conclusion is because there were few changes in tumor size and lymph node involvement across the samples because they were all at early stages of breast cancer (stages I and II). It was shown that in the group of patients with early-stage breast cancer, clinical-pathological variables did not significantly affect the patient’s prognosis in retrospective research by Jones et al. of 453 patients with stage I-II breast cancer. This study found no significant difference in the group with local recurrence compared to those without local recurrence. The small sample size was probably to blame for this outcome. Due to the high degree of heterogeneity in breast cancer, variations in the study’s sample size would impact the findings.

Breast cancer cells could protect themselves from unfavorable conditions, for example, when chemotherapy drugs are given. Breast cancer cells were originally epithelial cells transformed into malignant (EMT). An aberration in the cell themselves from unfavorable conditions, for example, when chemotherapy drugs are given. Breast cancer cells were originally epithelial cells transformed into malignant (EMT). An aberration in the cell signaling pathway triggered the process of EMT. Growth factors (such as TGF-β and wnt), transcription factors (snails, SMAD,
LEF, and nuclear β-catenin), cytoskeletal modulators (Rho family), and extracellular proteases (MMPs, plasminogen activators) are just a few of the signaling pathways that experienced deviations in expression, distribution, and function, leading to EMT. Other molecular changes will follow these initial changes; cytokertatin intermediate filament were replaced with vimentin. Vimentin functions as a protein building the framework of the cell and affects cell motility. Vimentin expression levels were associated with more aggressive cells, a greater capacity for metastasis, and a worse prognosis. Vimentin, actin microfilaments and microtubules are the three main components forming the cytoskeleton responsible for cell contraction and migration processes. According to research by Tam et al., protein kinase C α (PKCα) is used by EMT to switch between two kinase pathways. PKCα is activated upon EMT by the switch from autocrine platelet-derived growth factor receptor (PDGFR) signaling to EGF receptor signaling (EGFR) in mesenchymal stem-like cells and basal cell lines. PKCα activation will cause the transcription factor FRA1 (FOS-like antigen 1), which is necessary for the survival of cancer stem cells. On the other hand, TWIST, as an EMT marker was found to correspond to independent recurrence, and poor prognostic factor for breast cancer patients. Vimentin expression in this study differed significantly (p=0.000) between the groups that did not experience local recurrence and those that did. Whipple et al. discovered that regulation of vimentins is a reliable indicator of both metastatic disease progression and a dismal prognosis. The expression of vimentin was found to correlate with high microtentacl expression and decreased cytokertatin expression, both of which were frequently observed during the growth of tumors. Overexpression of an EMT marker was found to correspond with resistance to chemotherapy and recurrence of the disease, according to research from Mego et al. Two factors play an important role in breast cancer recurrence: the ability of cancer cells to regulate the microenvironment to protect them and the weakness or disruption of the body’s defense mechanism. A continuous inflammatory process will occur around the cancer cells in breast cancer patients. Inflammation caused by cancer cells will trigger the growth of cancer cells, among others, by weakening the immune system and stimulating angiogenesis and cell proliferation. Abundant cytokines were released by cancer during the malignancy process, and many studies showed that cytokines could promote carcinogenesis by both increasing the inflammation process and bringing up immunosuppression agents. Another cytokine that has a prominent role in the malignancy process is IL10. This cytokine has dual roles as an anti-inflammatory and as an immunoregulator. Breast cancer patients were shown to have high levels of IL10 expression, which suggests that IL10 has a pro-tumorigenic function in the development of the disease and is involved in tumor-induced immunosuppression. As inflammatory cytokines, IL10 was useful on one side by controlling the severity of autoimmune and inflammatory reactions; on the other, IL10 has a harmful role by weakening antitumor immunity and making the microenvironment favorable for tumor growth. IL10, formerly known as cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine that blocks T-cell/macrophage cytokine gene expression and synthesis and their ability to deliver antigens. It prevents the synthesis of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL1, IL11, IL6, IL8, IL12, IL18, and macrophage inflammatory protein-1α (MIP-1α). Like most other cytokines, IL10 affects various cells in different ways. This interleukin controls the host immunological response, making it more than just an anti-inflammatory cytokine. Through the activation of TH cells and peripheral blood mononuclear cells (PBMC), this cytokine also promotes the development of mast cells while suppressing IFN-γ. Additionally, B cell differentiation for immunoglobulin secretion is strongly stimulated by IL10. By reducing nuclear factor translocation kB (NF-kB), IL10 also blocks a pathway for immediate-early pro-inflammatory inhibition. According to this study, there was a significant difference in the expression of IL10 between the ILC group and the group that did not experience local recurrence within the first two years following surgery.
Apoptosis was brought on by various causes, including UV light, radiation, chemotherapy, and death receptor signaling. CD95-induced apoptosis is one of the widely recognized apoptosis pathways. A member of the TNF-R superfamily’s death receptor subfamily, CD95, belonged to that group. When CD95 is cross-linked with either its natural ligand, CD95L, or antagonistic antibodies, such as anti-APO-1, sensitive cells are put into apoptosis. When CD95 is activated, the death-inducing signaling complex (DISC) is produced. The DISC is composed of procaspase-8, procaspase-10, oligomerized CD95, the adaptor protein FADD that contains the death domain (DD), and c-FLIP (which has two splice variants, c-FLIPL and c-FLIPS). Due to the development of the CD95 DISC, procaspase-8 is autocatalytically cleaved at the DISC, resulting in active caspase-8 and starting the apoptotic signaling cascade.

According to the study’s findings, there was a statistically significant difference between the groups that experienced a local recurrence and those that did not (p=0.045) in the expression of CD95. By using a linear regression test, it was discovered that CD95 expression had a bad relationship with the chance of local recurrence in early-stage ILC. Similar results were reported by Strater et al., who discovered that tumor cells with low levels of CD95 expression had a poor prognosis and were more likely to recur. However, a high level of CD95 expression was associated with a prolonged period of disease-free after surgical treatment. In addition, Yamana and colleagues discovered that the presence of CD95 immunoreactivity in urothelial carcinoma was negatively connected with a greater pathological grade (P=0.0001), a more advanced stage of the tumor (P = 0.023), and a shorter overall survival time (P = 0.010). A low level of CD95 may result from the progression of cancer, which manifests itself most obviously with the dedifferentiation of cancer cells and is connected with cancer recurrence. The opposite result was shown in a study by Hoogwater, which said that high preoperative CD95L levels were associated with poor recurrence-free survival (RFS). The study also said that low-level preoperative CD95 is a potential good prognostic factor for patients undergoing colorectal surgery in patients who already have liver metastasis. This disparity is presumably attributable to the Hoogwater trial conducted on patients who had already undergone CD95 counterattack because their cancer had spread to the liver before the research was conducted.

Finally, the authors recognize that the study has limitations despite our homogeneous research population. The sample size for this cross-sectional study was limited, and the follow-up period was only a few months. The results of this study are restricted to demonstrating differences in expression between those who did not experience a recurrence and those who did because there is no recognized normal limit value for the expression of CD95. Carrying out investigations in the future to determine the percentage threshold of CD95 expression with a risk of recurrence (such as determining the expression of estrogen receptors and progesterone receptors) will be helpful so that CD95 expression can be used as a predictor factor for breast cancer recurrence.

CONCLUSION
Apoptosis evasion through inhibition of CD95 expression was important in the local recurrence of ILC after mastectomy and chemotherapy.

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CONFLICT OF INTEREST
The authors declare that no competing financial, professional, or personal interests might have affected the performance or
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ETHICAL APPROVAL

The study was conducted following the Declaration of Helsinki and approved by The Research Ethics Committee of the Faculty of Medicine, Universitas Jember, with number 1.537/H25.11.11/KE/2021.

REFERENCES


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