The New Ropanasuri Journal of Surgery

Volume 5 | Number 1 Article 1

6-10-2020

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Sonar Soni Panigoro
Division of Oncology Surgery, Department of Surgery, Faculty of Medicine Universitas Indonesia, dr. Cipto Mangunkusumo General Hospital, Jakarta, sonarpanigoro@gmail.com

Diani Kartini
Division of Oncology Surgery, Department of Surgery, Faculty of Medicine Universitas Indonesia, dr. Cipto Mangunkusumo General Hospital, Jakarta

Dewi Wulandari
Department of Clinical Pathology, Faculty of Medicine Universitas Indonesia, dr. Cipto Mangunkusumo General Hospital, Jakarta

Arif Supono
Division of Oncology Surgery, Department of Surgery, Faculty of Medicine Universitas Indonesia, dr. Cipto Mangunkusumo General Hospital, Jakarta

Recommended Citation

Panigoro, Sonar Soni; Kartini, Diani; Wulandari, Dewi; and Supono, Arif (2020) "Circulating Tumor Cell and Regulator T-Lymphocyte in Core Biopsy for Breast Cancer," *The New Ropanasuri Journal of Surgery*: Vol. 5 : No. 1 , Article 1.
DOI: 10.7454/nrjs.v5i1.1071
Available at: https://scholarhub.ui.ac.id/nrjs/vol5/iss1/1

The New Ropanasuri Journal of Surgery
OPEN ACCESS
Published by Department of Surgery
Faculty of Medicine, Universitas Indonesia

Circulating Tumor Cell and Regulator T-Lymphocyte in Core Biopsy for Breast Cancer

Sonar Soni Panigoro,[1] Diani Kartini,[1] Dewi Wulandari,[2] Arif Supono.[1]

1. Division of Oncology Surgery, Department of Surgery 2. Department of Clinical Pathology, Faculty of Medicine Universitas Indonesia, dr. Cipto Mangunkusumo General Hospital, Jakarta.

Corresponding author: arif.supono@gmail.com Received: 21/Apr/2020 Accepted: 05/Jun/2020 Published: 10/Jun/2020
Website: https://scholarhub.ui.ac.id/nrjs/ DOI:10.7454/nrjs.v5i1.1071

Abstract

Introduction. Core biopsy is one of the modalities used in breast cancer diagnosis, with a 91-99% sensitivity and specificity of 96-100%. The procedure causes tumor tissue damage to let tumor cells to enter circulation (CTC) and provoke the infiltration of inflammatory cells. Consider the side effect of core biopsy, the procedure to be debatable/contradicting opinions. Based on this premise, this study aims to investigate Tregs and CTC count in the core biopsy procedure.
Method. A study enrolled 32 blood sample from patients of Stage III and IV breast cancer who proceed core biopsy during August to December 2016. A consecutive sampling method employed in this study. Blood specimens taken two weeks before and after the procedure, and subjected to analyze of Tregs - (CD4, CD25, FoxP3) and CTC count (CK19) using flow cytometry. Wilcoxon test proceeded to analyze CTC/Tregs count and Spearman correlation test proceeded to find out the correlation of Tregs- and CTC count.
Results. A decrease of CTC after core biopsy found in median value of 50% in and $p = 0.569$. Median of Tregs count after core biopsy was 26.31%, with $p = 0.049$. We found a small rho values ($r = 0.165$, $r = 0.235$, $r = 0.046$, respectively) and $p > 0.0.5$.
Conclusion: No correlation between Tregs - with CTC count, before or after core biopsy. The study denoted that core biopsy considered a safe method for histopathological diagnostic purposes in breast cancer.

Keywords: breast cancer, core biopsy, Tregs, CTC

Introduction

Breast cancer is the most common malignancy in women in developed countries and second in developing countries. The incidence of breast cancer in Indonesia is highest compared to other malignancies. It estimated that there are 23.140 new cases each year. The majority of patients presented in advanced stages. The study of Ramli et al., in dr. Cipto Mangunkusumo General Hospital (RSCM), found that those of stage III were 43.4%, and stage IV was 14.3%. The mortality in 90% of patients associated with metastasis to vital organs.[1-3]
Core biopsy is one of the diagnostic modalities in breast cancer, which has a sensitivity of 91-99% and specificity of 96-100%. The procedure is relatively simple, however, the side effect which is the occurrence of CTC (circulating tumor cells) on 32% of cases, making this procedure to become a controversy/debate between experts.[4-8] Core biopsy causes damage to tissue and tumor blood vessels. Through the damaged vessels, the tumor cells released to circulation and known as CTC. An acute inflammatory process follows it with vasoamine release, degranulation of mast cells, which causes increased capillary permeability, and infiltration of inflammatory cells to the tumor area. In the final phase of infiltration, there is an increase in Tregs count (regulator T lymphocytes) which releases cytokines (TGF- β, IL-10).[5-10] Immunosuppression found in the microenvironment. TGF-β stimulates COX2, which stimulates autoregulation/cell conversion of CD4+ T lymphocytes to FoxP3+ T lymphocytes (Tregs) so that Tregs count increased. TGF-β stimulates SOX-4 (master EMT regulator), which causes EMT (Epithelial-Mesenchymal Transition) process. Tumor cells transform into an invasive mesenchymal phenotype, which penetrates vascular walls, and circulates in the blood as CTC. It can be observed that core biopsy causes an increase in Tregs count and CTC count.[5-10]
In contrast, other experts believed that core biopsy is a safe diagnostic procedure and does not cause the spreading of tumor cells through blood circulation.[11-14] The two contradicting opinions, as of the time of this present study, the safety of core biopsy remains a debate/controversy between experts, particularly in its reverse reaction that may induce an increase in CTC count. This study aims to determine whether there is a change and correlation between Tregs and CTC count before and after core biopsy.

CTC is the gate of cancer cells into circulation so that it could result in distant metastasis. CTC analysis from blood specimen has advantages over tissue biopsy because it can repeatedly proceed, less invasive, early metastasis detection, disease status, progressivity, therapeutical response, and more accurate prognosis prediction compared to other commonly used markers.[15,16] There are five types of CTC. 1. Traditional CTCs (intact cancer cells, viable nucleus, CK+); 2. CK- cells (stem cells/cancer cells that had undergone EMT); 3. Apoptotic CTCs (cancer cells that had undergone apoptosis); 4. Small CTCs (CK+ and CD45- cancer-specific biomarker); and 5. CTC clusters. High CTC count (>5 cells/7.5 mL blood) correlated with a poor progression-free and overall survival. CTC analysis uses antibody (CK19) to detect tumor cells of epithelial cells origin.[15,16]
Tregs are a subpopulation of T lymphocyte that plays a role in maintaining the innate immune system. T lymphocyte progenitor is

produced in the bone marrow and undergoes maturation in the thymus, which then differentiates into three types: 1. T-helper lymphocyte; 2. Cytotoxic-T lymphocytes; 3. T-regulatory (Tregs) lymphocytes. Identification and quantification of Tregs from CD4+ cells population proceeds using flow cytometry with CD4, CD25, and FoxP3 biomarkers. Normal Tregs count in the circulation should be no more than 0.4% of T cells population (CD4+), their presence in high proportions in tumors is generally a poor prognostic feature. Tregs suppresses the immune system to prevent immune reactions such as in autoimmune disease.[9,17,18] The ratio/balance of Tregs and T helper/effector (CD8+) lymphocytes determine whether a tumor is on progression/regression. If Tregs dominant, an immunosuppression microenvironment/process achieved. The immune system cannot detect tumor cells as foreign material; therefore, the tumor will progress. In contrast, if T helper/effector (CD8+) cells dominate, tumor cells will be recognized by the immune system and destroyed.[9,10,17]

Core needle biopsy causes damage to tumor tissue as well as blood vessels. Through damaged blood vessels, tumor cells released to the circulation (CTC). An acute inflammatory response followed, along with the release of complement-mediated, mast cell degranulation, vasoamine release, increased vascular permeability. During the tumor infiltration phase, infiltration of the inflammatory cells identified, including neutrophils, granulocytes, MSDCs, mast cells, monocytes, and macrophages. Tregs play a role in the later phase of infiltration to reduce the inflammatory process.[5,17]

The conversion of tumor-associated macrophages from M1 to M2 phenotype increases TGF-β. Autoregulation of Tregs addition achieved through the expression of COX2, which stimulates the conversion of CD4+ T lymphocytes into Tregs. Thus, Tregs will found increased, and producing an immunosuppressive microenvironment. MSDCs, Tregs, TGF-β, and IL-10 cytokines expression will also increase gradually. TGF-β stimulates the conversion of tumor-associated neutrophils from N1 (anti-tumor) to N2 tumor-associated neutrophils, which secrete CCL17 (pro-tumor), leading Tregs chemotaxis. Increased expression of TGF-β also stimulates SOX-4 activity, which is a master EMT regulator. EMT causes a change in property/shape of tumor cells into an invasive mesenchymal phenotype. We carried out a study aims to investigate Tregs and CTC count in the core biopsy procedure.

Method

The study implied a cross-sectional study in the Surgical Oncology Division of dr. Cipto Mangunkusumo General Hospital (RSCM) and Gatot Subroto Army Hospital (RSPAD), Jakarta, Clinical Pathology Laboratory of RSCM from August to December 2016. The population is all patients diagnosed as stage III and IV breast cancer managed in the two hospitals. A consecutive sampling method was used in the study. Those included were patients diagnosed as breast cancer stage III and IV who underwent core biopsy while those who previously treated and those who refuse to participate and drop out excluded.

Those who met the criteria were informed and signed the consent. Following the enrollment, an initial 5 mL blood specimen aspirated before biposy, a 2 mL processed for Tregs analysis, and another 3 mL for CTC analysis. Tregs count was measured using flow cytometry with CD24-FITC, CD25-PE, and Anti-FoxP3-APC antibody biomarker, and CTC count was measured using flow cytometry with CK19 (FITC) Santa Cruz (SC-53003) antibody biomarker. The analysis proceeded in Clinical Pathology Laboratory RSCM/FKUI. Two weeks after the procedure, another aspiration of blood specimen proceeded in the same manner.

Data in numeric described in mean with standard deviation for normally distributed ones, and median with 25 and 75 percentiles for non-normally distributed data; categorical data presented in percentage. The Wilcoxon– (normal distribution) and Spearman test (non-normal distribution) were used in the study to find out the correlation between Tregs and CTC count.

The Committee of Ethic, Faculty of Medicine, Universitas Indonesia approved the study No. 955/UN2.F1/ETIK/2016, November 7th 2016.

Results

Thirty-two patients met the criteria and enrolled in the study. The subjects in the study dominated with stage III (75%), and the most histopathology finding was invasive ductal carcinoma (81.3%). Whereas, tumor grade of most was intermediate grade (75%). A complete description of these characteristics presented in Table 1.

Table 1. Subjects' Characteristics

Variable	n = 32
Gender, n (%)	
Male	1 (3.1)
Female	31 (96.9)
Age (years), mean ± SD	46.46 ± 7.47
Stage, n (%)	
III	24 (75.0)
IV	8 (25.0)
Histopathology, n (%)	
IDC	26 (81.3)
DCIS	0 (0.0)
ILC	2 (6.3)
LCIS	0 (0.0)
Others	4 (12.5)
Grade, n (%)	
Low	2 (6.3)
Intermediate	24 (75.0)
High	6 (18.8)
Subtype, n (%)	
Luminal A	8 (25.0)
Luminal B	9 (28.1)
HER 2	4 (12.5)
TNBC	4 (12.5)
Others	7 (21.9)

Note: IDC: invasive ductal carcinoma; DCIS: Ductal carcinoma in situ, ILC: Invasive lobular carcinoma, LCIS: Lobular carcinoma in situ, HER2: human epidermal growth factor receptor 2, TNBC: triple-negative breast cancer.

The Tregs count after core biopsy expressed in median analyzed using the Wilcoxon test showed a decrease with p = 0.049, showing a significant correlation between core biopsy and decreasing Tregs count. While as, CTC count after core biopsy, which also expressed in median and also analyzed using the Wilcoxon test showed a decreasing CTC count with p = 0.569, which conveys into no significant correlation to core biopsy (Table 2). To this finding, it assumed that core biopsy did not lead to an increase of CTC nor Tregs count.

Table 2. Correlation of core biopsy to changes in CTC and Tregs count

Variable	Before	After	p
Tregs count, median (25^{th}-75^{th} percentile)	0.20 (0.10-0.43)	0.13 (0.05-0.26)	0.049
CTC count, median (25^{th}-75^{th} percentile)	8 (3-21)	4 (0-30)	0.569

Wilcoxon correlation test

Correlation between Tregs– and CTC count

Using the Spearman analysis, we found the correlation coefficient (rho) between Tregs– and CTC count before biopsy, after– , and delta CTC–Tregs was very low (r = 0.165, r = 0.235, r = 0.046, respectively) with p = 0.05 (p = 0.368, p = 0.195, and p = 0.804, respectively)(Table 3). To this finding, it assumed that no correlation between Tregs- and CTC count.

Table 3. Correlation of Tregs count and CTC count

Variable	r	p
CTC - Tregs Before	0.165	0.368
CTC - Tregs After	0.235	0.195
Delta CTC - Tregs	0.046	0.804

Spearman correlation test

Discussion

Of all twenty-four subjects with stage III breast cancer, twenty-two subjects showed positive CTC value before core biopsy (91%), while in eight subjects with stage IV, all showed positive CTC count before core biopsy (100%). A recent study of Yap et al.,[19] suggested that positive baseline CTC count correlated with poorer outcomes. Other previous studies also suggested that CTC may predict metastatic potential and a higher visceral tumor burden.[20] In patients with stage IV breast cancer, baseline CTC levels may be used as an independent predictor of progression-free and overall survival.[21] In an earlier study which compared the use of CTC count and imaging studies for prediction of overall survival in patients with metastatic breast cancer, assessment of CTC count found to be superior to imaging methods as CTC assay is more reproducible one, yields useful results at an earlier time point than imaging studies, and seems to be a more robust predictor of survival.[22]

The study showed a median of Tregs count before core biopsy was 0.19 out of 100 CD4+ cells, and the median of Tregs count after core biopsy was 0.14 out of 100 CD4+ cells. We found not increased but decreased. As data distributed not in the normal one, the Wilcoxon test carried out to analyze and found p = 0.049, denoting a significant difference of Tregs between before and after the core biopsy. However, this finding in contrast to a study of Edward et al. showing an increase in Tregs count in association to the increase of many critical inflammatory cytokines and mediators such as S100A8, CXCL1, CXCL2, IL-1b, TNF-a, and COX2.5 COX2 thought to stimulate the production of prostaglandin E2 (PGE2) which will in turn increase recruitment of EP-2 expressing FoxP3+ Tregs to the tumor via the PGE2/EP2 interaction-dependent pathway.[23] However, it should be noted that these preexisting studies were conducted in murine models.

The study findings showed that the median of CTC count before core biopsy was eight cells per 100.000 leukocytes, and CTC count after core biopsy was our cells per 100.000 leukocytes. We found no increase in CTC count after core biopsy. Again, as data distribution is not a normal one, the Wilcoxon test proceeded and showing p = 0.569. To this finding, it assumed that the core biopsy does not affect decreasing CTC count, in contrast to a study of Loughran CF (2011) that shows an increase in CTC after the core biopsy.[24] The difference in time of blood specimen collection may explain the phenomenon. After the final inflammation phase ends (approximately one week), Tregs count expected to increase, followed by proceeding phases until EMT and tumor escape phase occurs in approximately day 10. In this study, the second blood specimen collection proceeded fourteen days after core biopsy. The author expected to determine whether there is a prolongation of the inflammatory phase and tumor infiltration, where the tumor will continually release its cells to circulation. Another consideration for the timing of the procedure is that during the period, EMT and tumor escape phase is still ongoing and tumor cells that are detected are those who had passed EMT phase that is more resistant to immune surveillance, therefore, more clinically aggressive.[5,15,20] However, other studies showed that CTC is known to have a short survival time in the bloodstream, with an estimated range of 1 to 3 hours.[25] Other than that, increased CTC count occurs greatest in the first 24 hours after core biopsy in tumor infiltration phase.[15,20]

Table 2 showed that the median value of CTC and Tregs are both decreased after core biopsy. A Spearman correlation test carried out and a low rho value found (r = 0.165, r = 0.235, r = 0.046, respectively), each with a p-value of 0.368, p=0.195, and p=0.804, respectively. Our study suggested that there was no significant correlation between Tregs count and CTC count. Previous studies have suggested that Tregs count determines CTC count. Indeed, several factors may affect CTC count instead of CTC count. These factors are histopathological type, aggressivity of tumor cells/tumor grade, tumor stage, and immunological status.[12,24] Furthers, in the study of Ye et al., positive correlation between CTC count and Tregs ratio was observed in a lung cancer.[26]

The core biopsy is currently established as the most frequently used diagnostic method worldwide, mainly for malignant breast lesions. Some authors have observed tumor cells in needle tracts and blood and lymph vessels after core biopsy, while some others reported none in image-guided core biopsy.[27] The study demonstrated that core biopsy does not cause an increase in CTC nor Tregs count. However, it cannot be concluded that core biopsy is a safe diagnostic procedure because statistically significant results were only observed in Tregs reduction but not for CTC reduction. More extensive research with a better quantification method of CTC and Tregs and a large sample size is encouraged to elucidate the safety profile of core needle biopsy in breast cancer. It suggested that core biopsy remains a safe method for histopathological examination of breast cancer.

Conclusion

We conclude that core biopsy is considered a safe method for histopathological examination of breast cancer.

Disclosure

Author disclose no conflict of interests.

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